

REMARKS

Claims 1 and 32-52 were pending. Claims 32, 39, 43 and 46-48 have been canceled without prejudice herein. Claims 1, 33-38, 40-42, 44, 45, and 49-52 have been amended to more distinctly describe the claimed subject matter. New claims 53 and 54 have been added. No new matter has been added. Support for the amendments can be found throughout the specification and claims as originally filed. Specifically, support for new claims 53 and 54 can be found in previously presented and examined claims 1, 33-38, 40, and 41.

Accordingly, upon entry of the present amendment, claims 1, 33-38, and 40-42, 44, 45, 49-54 will be pending in the application.

Any amendments to and/or cancellation of the claims are not to be construed as an acquiescence to any of the rejections set forth in the instant Office Action, and were done solely to expedite prosecution of the application. Applicants hereby reserve the right to pursue the subject matter of the claims as originally filed in this or a separate application(s).

No additional search is required and no new issues have been raised by the amendments made herein. Furthermore, in view of the amendments and arguments set forth herein, the number of claims pending and the issues for appeal have been reduced. It is believed that the Examiner's rejections under §101 and §112 have been obviated by the claim amendments and cancellations herein. Therefore, the claim amendments and cancellations made herein are permissible under 37 C.F.R. §1.116 as reducing the number of issues for appeal, and Applicants respectfully request that the present Amendment be entered.

Claim Objections

The Examiner states that the steps in claim 49 should be labeled a-e. The claim has been amended to effect this change thereby obviating this objection.

The Pending Claims

The pending claims are directed to a mouse comprising in its genome a first exogenous DNA molecule that functionally disrupts a NFATp gene of said mouse and a second exogenous DNA molecule that functionally disrupts a NFAT4 gene of said mouse, *wherein said mouse exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production relative to a wildtype mouse*. The claims are further directed to a method for identifying a test

compound that modulates Th2 cell activation via a pathway that does not *directly* modulate NFATp or NFAT4 comprising: a) administering said test compound to a first mouse comprising a genome deficient in NFATp and NFAT4; b) administering an appropriate control compound to a second mouse comprising a genome deficient in NFATp and NFAT4, ***wherein the phenotypes of the first transgenic mouse and the second transgenic mouse are characterized by increased Th2 cytokine production***; and c) evaluating Th2 cell activity in said first mouse relative to Th2 cell activity in said second mouse wherein a change in Th2 cell activity in said first mouse relative to Th2 cell activity said second mouse identifies a compound as one that regulates Th2 cell activation via a pathway that does not *directly* modulate NFATp or NFAT4. The claims are further directed to a method for producing a mouse lacking NFATp and NFAT4, ***wherein said mouse exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production relative to a corresponding wild-type mouse***, comprising: (a) introducing an exogenous DNA molecule comprising at least a portion of a NFATp gene into a mouse embryonic stem cell to create a first modified stem cell in which the endogenous NFATp gene of the embryonic stem cell is functionally disrupted; (b) introducing said first modified stem cell into a mouse such that said mouse produces at least one offspring comprising a functionally disrupted NFATp gene; (c) introducing an exogenous DNA molecule comprising at least a portion of a NFAT4 gene into a mouse embryonic stem cell to create a second modified stem cell in which such that the endogenous NFAT4 gene of the embryonic stem cell is functionally disrupted; (d) introducing said second modified stem cell into a mouse such that said mouse produces at least one offspring comprising a functionally disrupted NFAT4 gene; and (e) mating said at least one offspring with a functionally disrupted NFATp gene with said at least one offspring with a functionally disrupted NFAT4 gene and identifying subsequent offspring with both a functionally disrupted NFATp gene and a functionally disrupted NFAT4 gene ***to thereby produce a mouse lacking NFATp and NFAT4 which exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production relative to a wild-type mouse***. The claims are further directed to a method for producing a transgenic mouse with a functionally disrupted NFATp gene and a functionally disrupted NFAT4 gene, ***wherein said mouse exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production***, comprising: mating a mouse having a functionally disrupted NFATp gene with a mouse having a functionally disrupted NFAT4 gene and identifying subsequent progeny with both a functionally disrupted NFATp gene and a functionally disrupted NFAT4 gene, to thereby produce a mouse with a functionally disrupted

NFATp gene and a functionally disrupted NFAT4 gene that ***exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production***. The claims are also directed to a mouse comprising in its genome a first exogenous DNA molecule that functionally disrupts a NFATp gene of said mouse and a second exogenous DNA molecule that functionally disrupts a NFAT4 gene of said mouse, ***wherein said mouse exhibits a non-wildtype phenotype characterized by increased Th2 cytokine production***, blepharitis, interstitial pneumonitis splenomegaly and lymphadenopathy, and increased levels of serum IgG1 and IgE, relative to a wildtype mouse. ***Accordingly all of the claims are directed to non-wildtype mice or methods of using such mice or cells isolated therefrom.***

Claim Rejections Under 35 USC §101

Claims 1 and 32-51 have been rejected under 35 USC §101 because the Examiner is of the opinion that the claims are not supported by a specific or substantial asserted utility. This rejection is respectfully traversed.

The Supreme Court has held that a new product or process must be shown to be ‘operable’ (*i.e.*, that it can be used to effect the proposed) in order to meet the utility requirement. *Mitchell v. Tilghman*, 86 U.S. (19 Wall.) 287,396 (1873). The U.S. Patent and Trademark Office bears the initial burden of challenging an applicant’s asserted utility. Specifically, in *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995), it was held that:

Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention’s asserted utility.

In addition, the Federal Circuit has held that “[t]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding a lack of utility.” The Examiner has not met the standard required to shift the burden to the Applicants to convince one of skill in the art that the claimed invention is useful.

The Examiner states that claims 1 and 32 do not require that the mouse have a non-wild-type phenotype. Claim 32 has been canceled without prejudice herein thereby obviating the rejection with respect to this claim. The rejection is traversed on the grounds that claim 1 specifically requires that the mouse is a *exhibit a non-wild type phenotype characterized by increased Th2 cytokine production relative to a wildtype mouse*. Contrary to the Examiner's statement, this phrase is not in the preamble of the claim, it is in the body of the claim. Accordingly, the claim *requires* a phenotype different from a wildtype mouse. The Examiner states that "the phrase does not bear patentable weight because it may not occur." However, the claim requires that the result occur and it is not clear why the Examiner asserts that it may not occur. Applicants' provide a working example which demonstrates that functional disruption of both NFATp and NFAT4 results in a mouse with this non-wildtype phenotype. Accordingly, the mice of claim 1 exhibit a non-wildtype phenotype.

The phenotype of these knockout mice is useful. The Examiner asserts that "[t]he art at the time of filing did not teach the NFAT4 or NFATp deficient mice were a model for disease or teach how to use the mice to screen," and further asserts that "[t]he art since the time of filing does not teach the mice are a model for disease or teach how to use the mice to screen compounds." Applicants point out that this review of the state of the art at the time of and since filing is misplaced without concurrent review of the teachings of the specification. The Examiner seems to suggest that the instant invention is of such limited utility that it does not meet the requirements for patentability. Applicants point out that such a review of the art cannot form the basis for a determination of lack of utility. Rather, the claimed invention must be evaluated to determine whether it is useful for at least one purpose described in the specification. The instant specification teaches that mice deficient in NFATp and NFAT4 have a readily detectable phenotype characterized by increased Th2 cytokine production. Consequently, as taught in the specification, the mice and lymphoid cells derived from these mice are particularly useful in methods for identifying modulators of Th2 cytokine production. Moreover, increased Th2 cytokine production is a model for several human disorders. For example, the specification teaches that reducing Th2 cytokine production is desirable in cases such as allergies (to thereby downregulate IgE production) and infectious diseases and cancers, in which biasing of the immune response to a Th1 type response may be beneficial (see page 30, lines 31-39). In addition, increased Th2 cytokine production has been associated with atopic asthma (see, *e.g.*,

Ying *et al.* 1995. *Am. J. Respir. Cell Mol. Biol.* 12:477 and Robinson *et al.* 1992 *N. Eng. J. Med.* 326:298; attached as Appendices A and B) and with ulcerative colitis (see Boirivant *et al.* 1998. *J. Exp. Med.* 188:1929; attached as Appendix C). Accordingly, it is accepted that decreasing Th2 cytokine production would be of benefit in treatment of certain disorders.

The Examiner further states that “[t]he specification further contemplated the mouse to identify agents that modulate Th2 cell activity by means other than modulating NFATp or NFAT4 (pg 10, lines 31-34). Such a use is not substantial.” Once again the Examiner has not met his burden of showing that one of ordinary skill in the art would reasonably doubt the utility asserted by these claims. While the Examiner may be of the personal opinion that the claimed invention lacks utility, no evidence has been presented to support this position. The instant application clearly teaches that mice lacking NFATp and NFAT4 have increased Th2 cytokine production compared to wildtype mice. The specification further teaches that mice deficient in NFAT4 and NFATp are useful for identifying compounds that reduce Th2 cytokine production because of their easily identifiable phenotype. As set forth above, it is also clear that increased Th2 cytokine production is associated with several human disease states. Accordingly, these animals are clearly useful to identify compounds that would reduce Th2 cytokine production. Such compounds would potentially be of great benefit in treating patients suffering from these disorders. The fact that it may also possible to identify compounds that reduce Th2 cytokine production by assaying for compounds that directly act on NFATp or NFAT4 is not relevant to the determination of the utility of the claimed invention.

Accordingly, it is respectfully requested that the rejection of claims 1 and 32-51 under 35 U.S.C. §101 as it may be applied to any of the presently pending claims be reconsidered and withdrawn.

Claim Rejections Under 35 USC §112, First Paragraph

Claims 1, 32-51, and 52 have been rejected under 35 USC §112, first paragraph as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner also states that

the claimed invention has no specific utility and therefore one skilled in the art would not know how to use the claimed invention. The Examiner further contends that claims 32-51 and 52 do not convey to one of skill in the relevant art that “the inventor(s), at the time the application was filed, had possession of the claimed invention.”

With respect to enablement, as set forth above, the Court in *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995), held that:

Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention’s asserted utility.

Additionally, the Court stated that in the absence of a reason to doubt the objective truth of the teachings contained in the specification, the methods of making and using the claimed invention must be taken as complying with the requirements of §112, first paragraph. As set forth above, the claimed invention is useful and, therefore, in the absence of any reason provided to doubt the objective truth of the teachings in the specification, the enablement requirements of §112, first paragraph must be found to have been met.

With respect to the written description rejections made by the Examiner, applicants point out that the phenotype in claim 39 is not new matter. Claim 39 is directed toward a double transgenic mouse with a phenotype characterized by compromised FasL expression leading to defective apoptosis. At page 38, lines 10-13, the specification states “Northern blot analysis revealed nearly complete absence of FasL transcripts in DKO T cells after 6 hours stimulation with anti-CD3. We conclude that the massive splenomegaly and lymphadenopathy observed in DKO mice is due at least in part to compromised FasL expression and defective apoptosis over time.” Nonetheless, in the interest of expediting prosecution of the application, claim 39 has been canceled.

With respect to claim 4, the Examiner states that there is support for “identifying agents that modulate Th2 cell activity by means other than modulating NFATp or NFAT4 themselves.”

The claim has been amended to include the term “directly” to more particularly point out and more distinctly claim the invention.

The written description rejection of claims 47 and 48 has been rendered moot by the cancellation of these claims and the written description rejection of claim 49 has been addressed by amendment of the claim to include the term “endogenous.”

With respect to claim 51, the term “fertilized egg cells” has been removed from the claim thereby obviating the rejection.

With respect to claim 45, Applicants point out that the specification does teach compounds that modulate immune cell activation. Specifically, Th2 cytokines are taught in the specification and are known in the art to modulate activation of immune cells.

Accordingly, it is respectfully requested that the rejection of claims 1 and 32-52 under 35 U.S.C. §112, first paragraph as it may be applied to any of the presently pending claims be reconsidered and withdrawn.

Claim Rejections Under 35 USC §112, Second Paragraph

Claims 1 and 32-51 have been rejected under 35 USC §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner asserts that “[t]he metes and bounds of what applicants consider “memory/activated phenotype” (claim 38) remains indefinite.” This rejection is respectfully traversed, however in the interest of expediting prosecution, Applicants have amended this claim to specifically recite that the peripheral T cells of the mouse have a higher percentage of memory/activated characterized by an increase in the levels of Mel-14 and CD45RB, and a decrease in the levels of CD44 and CD69.

With respect to claim 43, The Examiner argues that the term “IL-4 dependent immunoglobulin isotypes” is unclear. This rejection is respectfully traversed. Applicants point the Examiner to page 6, lines 10-13 which teaches that IL-4 dependent immunoglobulin isotypes

are those that are associated with efficient B cell help such as IgG1 and IgE, two isotypes known in the art to provide that help to B cells:

As used herein, the term "Th2 cell activity" refers to activity of a subpopulation of CD4+ T cells that is characterized by the production of one or more cytokines selected from IL-4, IL-5, IL-6, IL-10 and IL-13, and that is associated with efficient B cell "help" provided by the Th2 cells (e.g., enhanced IgG1 and/or IgE production).

However, in the interest of expediting prosecution of the application, claim 43 has been canceled.

The rejection of claim 48 has been rendered moot by the cancelation of that claim.

The Examiner further argues that "claims 49 and 52 are not directed toward a transgenic mouse." This rejection is respectfully traversed. Claims 49 and 52 are directed to methods of making non-wildtype mice that lack NFATp and NFAT4 and exhibit increased Th2 cytokine production relative to wildtype mice. The term "transgenic" has been removed from the claims to eliminate any confusion as to what the claims are directed.

The Examiner finds claim 49 indefinite because "female mice are not 'pseudopregnant.'" Applicants contend that the term "pseudopregnant" was well known in the art at the time of filing the application and accurately described the state of the mice as treated using art recognized techniques prior to implantation. However, in the interest of expediting prosecution of the application, the term has been removed from the claims.

With respect to claims 50 and 51, the Examiner states that these claims "are indefinite because the phrase 'mouse transgenic cell' is unclear. . . as it is unclear if the phrase is limited to cells isolated from a transgenic mouse or if the phrase encompasses any mouse cell that has been genetically altered so that it has a disrupted NFATp gene and a disrupted NFAT4 gene." This rejection has been obviated by the amendment to claim 50 to recite "an isolated cell from the mouse of claim 1."


Accordingly, it is respectfully requested that the rejection of claims 1 and 32-51 under 35 U.S.C. §112, second paragraph as it may be applied to any of the presently pending claims be reconsidered and withdrawn.

SUMMARY

Reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the examiner is urged to call the undersigned at (617) 227-7400.

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Respectfully submitted,

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Phenotype of Cells Expressing mRNA for TH2-Type (Interleukin 4 and Interleukin 5) and TH1-Type (Interleukin 2 and Interferon γ) Cytokines in Bronchoalveolar Lavage and Bronchial Biopsies from Atopic Asthmatic and Normal Control Subjects

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We investigated the phenotype of cells expressing messenger RNA encoding interleukin 4 (IL-4), IL-5, IL-2, and interferon γ (IFN- γ) in bronchoalveolar lavage (BAL) and bronchial biopsies (BX) from seven mild atopic asthmatic patients and nine nonasthmatic controls. Immunocytochemistry followed by *in situ* hybridization using either 35 S- or digoxigenin-labeled riboprobes was performed on cytopins from BAL and BX, respectively. With BAL or BX, *in situ* hybridization alone showed significant increases in percentages of IL-2, IL-4, and IL-5 mRNA⁺ cells when asthmatics were compared to nonasthmatic controls. Double immunocytochemistry-*in situ* hybridization revealed that > 70% of IL-4 and IL-5 mRNA⁺ cells were activated T cells (CD3⁺). The remaining IL-4 and IL-5 mRNA⁺ signals were colocalized to tryptase⁺ mast cells, and activated eosinophils (EG2⁺). Rare IL-4 and IL-5 mRNA⁺ cells were observed in nonasthmatic controls, the majority being CD3⁺ cells, as were IL-2 and IFN- γ mRNA⁺ cells (in both asthmatics and controls). A few IL-4 (< 8%) and IL-5 (< 5%) mRNA⁺ signals did not colocalize with any of the cells identified by immunocytochemistry. Thus, we provide further evidence that CD3⁺ T cells are the most abundant cells expressing IL-4 and IL-5 mRNA in BAL and BX from allergic asthma. Fewer, but detectable, numbers of tryptase⁺ mast cells and EG2⁺ eosinophils also expressed these transcripts.

Studies using bronchoalveolar lavage (BAL) cells and bronchial biopsies (BX) have shown that atopic asthma is characterized by local infiltration of activated T cells (CD25⁺) and eosinophils (EG2⁺) (1-3). Their numbers could be related to disease severity, and significant correlations were observed between the numbers of CD4⁺/CD25⁺ and EG2⁺ cells, compatible with the hypothesis that activated T cells are involved in eosinophil recruitment (4-6). It is now well established that even in humans, helper T cells can be broadly divided into two functional subsets, termed type 1 (TH1) and type 2 (TH2), according to the profile of cytokines they elaborate (7-9). TH1 cells predominantly secrete interleukin 2 (IL-2) and interferon γ (IFN- γ), but little IL-4 and

IL-5, and mediate delayed-type hypersensitivity, whereas TH2 cells elaborate predominantly IL-4 and IL-5, but little IL-2 and IFN- γ , and participate in helminthic infection and atopic disease (10-13).

Interleukin 4 is responsible for B cell isotype switching in favor of immunoglobulin E (IgE) production (14). Recent studies have shown that IL-4 also enhances expression of the adhesion molecule VCAM-1 on the surface of endothelial cells (15, 16). Because VCAM-1 participates in selective eosinophil adhesion via a VLA-4-dependent mechanism, IL-4 is not only pivotal in local IgE regulation but may account, at least in part, for selective eosinophil recruitment at sites of allergic tissue reactions. IL-5 promotes the terminal differentiation of committed eosinophil precursors, activates mature eosinophils, and prolongs their survival in culture and, by implication, at sites of allergic inflammation (17-19). IL-5 also selectively enhances eosinophil degranulation, antibody-dependent cytotoxicity, and adhesion to vascular endothelium (20, 21). Thus, IL-5 is strongly implicated in promoting selective accumulation and activation of eosinophils in asthma and allergic disease. Using the techniques of *in situ* hybridization (ISH) and immunocytochemistry (ICC), we previously identified an increase in the number of cells expressing mRNA encoding TH2-like cytokines in allergen-induced late-phase cutaneous and nasal reactions, and in BAL cells and BX obtained from atopic asthmatics (22-25). We also observed correlations between

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Abbreviations: alkaline phosphatase antialkaline phosphatase, APAAP; bronchoalveolar lavage, BAL; bronchial biopsies, BX; activated T cells, CD25⁺; activated eosinophils, EG2⁺; immunocytochemistry, ICC; immunoglobulin, Ig; interleukin, IL; interferon γ , IFN- γ ; *in situ* hybridization, ISH; phosphate-buffered saline, PBS; paraformaldehyde, PF; T-helper cells, TH.

TABLE 1
Clinical characteristics of atopic asthmatic and nonasthmatic control subjects

| Subject | Age | Sex | Allergen* | | | FEV ₁ (% predicted) | Methacholine PC ₂₀ (mg/ml) | Total IgE (IU/ml) |
|---------|-----|-----|-----------------|--------------------|-----|-----------------------------------|---|----------------------|
| | | | Grass Pollen | House Dust Mite | Cat | | | |
| Asthma | | | | | | | | |
| 1 | 28 | M | + | + | — | 95.9 | 1.2 | 259 |
| 2 | 26 | M | + | + | — | 88.7 | 6.5 | 268 |
| 3 | 43 | M | — | + | — | 89.3 | 2.4 | 645 |
| 4 | 37 | M | — | + | + | 75.7 | 2.0 | 143 |
| 5 | 33 | F | + | + | + | 96.9 | 7.4 | 209 |
| 6 | 44 | M | — | + | + | 88.3 | 6.2 | 89 |
| 7 | 28 | M | — | + | + | 91.2 | 6.5 | 259 |
| Control | | | | | | | | |
| 1 | 27 | F | — | — | — | 109.8 | >32 | 9 |
| 2† | 27 | M | + | + | — | 97.0 | >32 | 14 |
| 3 | 33 | F | — | — | — | 109.5 | >32 | 16 |
| 4 | 24 | M | — | — | — | 103.3 | >32 | 279 |
| 5 | 27 | M | — | — | — | 113.5 | >32 | 29 |
| 6 | 26 | M | — | — | — | 110.2 | >32 | 44 |
| 7 | 23 | M | — | — | — | 95.5 | >32 | 79 |
| 8 | 19 | M | + | — | — | 108.5 | >32 | 720 |
| 9 | 19 | M | — | — | — | 88.6 | >32 | 24 |

* Allergen sensitivity was determined by skin-prick test and radioallergosorbent test. Plus signs indicate positive results, and minus signs, negative results.
† "Normal" atopic subject.

the numbers of activated EG2⁺ and mRNA expression for IL-4 and IL-5. Although IL-4 and IL-5 are known to be secreted by activated T lymphocytes, recent studies have suggested that other cell types, including mast cells and eosinophils (26–29), may also transcribe mRNA encoding these cytokines. Thus, an appreciation of the precise cellular origins of these cytokines is fundamental to an understanding of the pathogenesis of atopic asthma.

Asthma pathogenesis is typically investigated by examining cellular changes in BAL fluid and BX. Although broad correlations have been demonstrated between the cellular changes in BAL and biopsies in asthma (30), it is by no means clear that either technique accurately samples relevant airway cells. Biopsies may provide static "snapshots" of the inflammatory process within the asthmatic bronchial mucosa, whereas BAL may be more sensitive to the dynamics and function of cells migrating into the airway (30). Thus, BAL and biopsy studies likely complement but do not supersede each other in the study of asthma. For these reasons, in the present study, we compared the amount and cellular source of cytokine expression in BAL and BX obtained contemporaneously from a group of mild atopic asthmatic patients and normal, mostly nonatopic controls. For this purpose, we developed a method combining *in situ* hybridization and ICC (31–33) to identify the phenotype of cells expressing mRNA for IL-4, IL-5, IL-2, and IFN- γ .

Materials and Methods

Subjects

Seven atopic asthmatic patients were recruited from the Allergy Clinic of the Royal Brompton Hospital, London, England. Nine nonasthmatic controls (7 nonatopic, 2 atopic nonasthmatics) not suffering from atopic disease and not taking any medication were recruited from staff of the Royal

Brompton Hospital and National Heart and Lung Institute. Informed written consent was obtained, and the study was approved by the local ethics committee. Clinical and demographic characteristics of the subjects are shown in Table 1. Asthmatic patients with seasonal symptoms were studied out of the United Kingdom's pollen season. One cat-allergic asthmatic patient, but none of the control subjects, kept a cat. The asthmatic patients had mild symptoms requiring only intermittent inhaled β_2 -agonist therapy. All were nonsmokers and none had received oral or inhaled corticosteroid therapy in the 3 months prior to the study.

Fiberoptic Bronchoscopy

Bronchoscopy for BAL and BX was performed in controls and subjects with asthma by the same operator (S.R.D.) as previously described (23, 25). Biopsies were taken from the lower and segmental bronchi of the right lower, middle, and upper lobes.

Processing of BAL Cells and BX

Bronchial biopsies and BAL were processed as described (23, 25). Briefly, cytopins were prepared from BAL cells (at a concentration of 0.5×10^6 cells/ml) using 0.1% poly-L-lysine (Sigma, Poole, UK)-coated RNase-free slides with a Shandon 2 Cytospin device (Shandon Southern Instruments, Runcorn, UK). Samples were air-dried for 10 min, fixed in 4% paraformaldehyde (PF) in phosphate-buffered saline (PBS; pH 7.4) for 30 min, and washed with 15% sucrose (Sigma) in PBS. Slides were incubated at 37°C overnight, then stored at -80°C until used.

Bronchial biopsies were fixed in 4% PF/PBS for 2 h followed by washing in 15% sucrose/PBS twice, then snap-frozen in isopentane (BDH, Leicester, UK) cooled in liquid nitrogen stored at -80°C .

Preparation of Riboprobes

Radiolabeled (Sulphur-35; Amersham International, Amersham, UK) and nonradiolabeled (digoxigenin; Boehringer Mannheim, Mannheim, Germany) riboprobes (sense and antisense) encoding cytokines IL-4, IL-5, IL-2, and IFN- γ were prepared as previously described (22, 23, 31, 32).

Immunohistochemical Staining

Bronchoalveolar lavage cytopins (-80°C) were warmed to room temperature. Cryostat sections ($6\text{ }\mu\text{m}$) were freshly cut from BX, mounted on 0.1% poly-L-lysine-coated slides, and air-dried overnight at 37°C .

Immunostaining using the alkaline phosphatase antialkaline phosphatase (APAAP) technique was performed first on BAL cytopins and BX sections using monoclonal antibodies directed against human T cells (CD3; Becton Dickinson, Cowley, Oxford, UK), activated eosinophils (EG2⁺, which recognizes the secreted form of eosinophil cationic protein; Pharmacia, Uppsala, Sweden), macrophages (CD68; Dako, High Wycombe, UK), and human mast cells (tryptase; Chemicon International Inc., Temecula, CA) (4, 31, 32). The details of ICC were described previously (31-33). For negative control preparation, the primary antibody was replaced with either nonspecific mouse Ig or Tris-buffered saline (TBS). Ten BAL cytopins and 10 biopsy sections were stained for each cellular marker, two of which were subsequently used for ISH with each probe. Furthermore, two BAL cytopins and two biopsy sections were performed for ISH alone.

In Situ Hybridization

After immunohistochemical staining of BAL cells or BX sections on slides as described previously, the specimens were immediately refixed by immersion in 4% PF/PBS for 5 min and permeabilized by immersion in 0.3% Triton X-100 in PBS for 10 min. After a brief wash in PBS, specimens were then further permeabilized by exposure to proteinase K (Promega, Southampton, UK) solution ($1\text{ }\mu\text{g/ml}$ in 20 mM Tris-HCl and 1 mM EDTA, pH 7.2) for 10 min at 37°C , the activity of which was terminated by immersion in 4% PF/PBS for 5 min. After a brief rinse in PBS, slides were air-dried.

For BAL cytopins, the procedure of ISH was performed as before (33). Applying this procedure, ICC-positive cells were stained red, whereas ISH-positive signals were localized as dense collation of silver grains overlying cells (black grains under bright field, which appear as white grains under dark field). As positive control for IL-4, IL-5, and IL-2, cytopins from a peripheral blood T-lymphocyte clone obtained from a patient with the hyper-IgE syndrome were prepared (22). Phytohemagglutinin (PHA)-stimulated blood mononuclear cells were used for IFN- γ -positive control (22). In addition, cytopins prepared from human IL-5 gene-transfected Cos-7 cells were also used for IL-5-positive control. For negative controls, slides were hybridized to sense probes and/or pretreated with RNase A, then hybridized to antisense probes (22, 25, 33).

For bronchial sections, the method has been described in detail elsewhere (31, 32). Using this method, the cellular phenotypic markers were stained red, whereas mRNA-

positive signals were shown as dark blue. Double-positive cells exhibited a mixed red and dark or purple color. In addition to positive and negative controls mentioned earlier, several other negative control experiments were performed as follows: a) performing ISH experiments without previous ICC, b) ICC alone (no ISH), c) omitting the probe in ISH protocol or using sense digoxigenin-labeled RNA probes for IL-4, IL-5, IL-2, and IFN- γ , d) pretreatment of sections with RNase A before ISH, e) use of an unrelated antisense digoxigenin-labeled RNA probe (rat insulin), and f) omitting the sheep polyclonal antidigoxigenin conjugated with alkaline phosphatase.

Quantitation of Numbers of Cells Expressing Cytokine mRNA and Phenotypic Markers

In BAL cytopins, at least two slides were stained for each cell phenotypic marker and at least two for each phenotypic marker subsequently probed for expression of each cytokine mRNA. Hybrids between cytokine mRNA and cRNA probes were localized as dense collections of silver grains in photographic emulsion overlying individual cells. Because it was not possible to count individual silver grains, cells expressing cytokine-specific mRNA were quantified in terms of the numbers of cells with overlying silver grains per 1,000 BAL cells in the cytospin preparation. The slides were counted by two independent observers using a Zeiss microscope (Göttingen, Germany) with bright and dark fields. For each phenotypic marker and cRNA probe, the coefficient of variability of the differences between the cell counts obtained from all slides by each observer did not exceed 5%.

With BX, the numbers of positive cells with ICC markers, ISH probes, and double ICC/ISH were counted in a blinded manner in coded random order by two observers. Two methods were used in biopsies. In the first, all cells in individual biopsies were counted and cell numbers expressed per square millimeter of area of the biopsies using a computerized program (Apple IIe; Apple Computer, Inc., Cupertino, CA). In the second, cells were counted to a depth of 0.45 mm below the basement membrane, and the numbers of cells were expressed per millimeter of length of basement membrane. In addition, for each specimen, the percentages of cells expressing cytokine-specific mRNA that coexpressed cellular phenotypic markers were also enumerated. The coefficient of variability of the differences between the counts obtained from all slides by both observers was $< 5\%$.

Analysis

Data were analyzed using a statistical package (Minitab Release 7; Minitab Inc., State College, PA). All between-subject and within-subject comparisons were analyzed by use of the Mann-Whitney *U* test. Correlation coefficients were obtained by Kendall's rank method with correction for tied values. *P* values < 0.05 were considered significant.

Results

Bronchoalveolar lavage cells and BX were obtained from seven atopic asthmatic patients and nine nonasthmatic controls. The total volumes of BAL fluid recovered from the asthmatic and control subjects were not significantly different (median 120 ml versus 130 ml, $P > 0.05$). The absolute

TABLE 2
Absolute numbers of total cells ($\times 10^5/\text{ml}$) and CD3⁺, CD68⁺, EG2⁺, and tryptase⁺ cells ($\times 10^4/\text{ml}$) in BAL fluid from atopic asthmatic and normal control subjects

| No. | Cells/ml | CD3 | CD68 | EG2* | Tryptase |
|----------------|----------------|-----------------|---------------|---------------------|--------------------|
| Asthma | | | | | |
| 1 | 0.7 | 1.3 | 5.1 | 0.2 | 0.006 |
| 2 | 1.0 | 1.5 | 7.0 | 1.2 | 0.002 |
| 3 | 0.8 | 0.9 | 6.1 | 0.8 | 0.005 |
| 4 | 1.2 | 1.3 | 8.8 | 1.5 | 0.02 |
| 5 | 0.8 | 0.8 | 6.4 | 0.4 | 0.01 |
| 6 | 1.1 | 2.1 | 7.3 | 0.6 | 0.01 |
| 7 | 0.8 | 3.3 | 4.0 | 0.6 | 0.01 |
| Mean \pm SEM | 0.9 \pm 0.07 | 1.6 \pm 0.3 | 6.4 \pm 0.6 | 0.8 \pm 0.17 | 0.009 \pm 0.002 |
| Control | | | | | |
| 1 | 0.7 | 1.0 | 5.5 | 0.00007 | 0.004 |
| 2 | 0.9 | 0.7 | 7.9 | 0.00009 | 0.01 |
| 3 | 0.9 | 1.4 | 7.1 | 0.004 | 0.01 |
| 4 | 0.7 | 0.7 | 5.6 | 0.003 | 0.01 |
| 5 | 0.6 | 0.6 | 5.0 | 0.00006 | 0.002 |
| 6 | 0.7 | 0.7 | 5.9 | 0.0 | 0.004 |
| 7 | 1.0 | 1.6 | 7.8 | 0.0 | 0.01 |
| 8 | 0.8 | 0.8 | 6.7 | 0.0 | 0.01 |
| 9 | 1.0 | 1.2 | 8.0 | 0.001 | 0.01 |
| Mean \pm SEM | 0.8 \pm 0.05 | 0.97 \pm 0.12 | 6.6 \pm 0.4 | 0.0009 \pm 0.0005 | 0.0078 \pm 0.001 |

* $P < 0.001$.

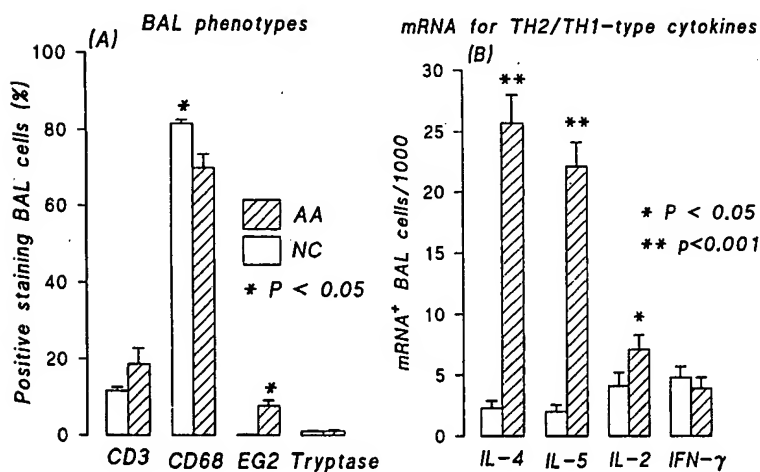


Figure 1. A comparison of BAL cells from atopic asthmatic patients (AA) and normal nonasthmatic controls (NC) using either immunocytochemistry alone (A) for phenotypic markers or *in situ* hybridization alone (B) for cytokine mRNA⁺ cells. AA, $n = 7$. NC, $n = 9$.

numbers of total cells and cells expressing the phenotypic markers CD3, CD68, EG2, and tryptase are shown in Table 2. There was no significant difference in total cell recovery between asthmatic patients (mean 0.9, range 0.8 to 1.2×10^5 cells/ml) and normal controls (mean 0.8, range 0.6 to 1.0×10^5 cells/ml). Whereas the absolute numbers of EG2⁺ eosinophils were significantly elevated in the asthmatic patients as compared with the controls ($P < 0.001$, Table 2), the absolute numbers of CD3⁺, CD68⁺, and tryptase⁺ cells were not statistically different. ICC and ISH were performed both separately and consecutively.

BAL Cells

Slides were first assessed for CD3, CD68, EG2, and tryptase immunoreactivity using the APAAP technique. Compared

with nonasthmatic controls, the asthmatic patients had increased percentages of EG2⁺ eosinophils ($P < 0.05$) and reduced percentages of CD68⁺ macrophages ($P < 0.05$) (Figure 1A). Because the absolute numbers of CD68⁺ macrophages did not differ in the asthmatic and the control subjects, this reduced percentage of macrophages in the asthmatics must be attributable to the increased absolute numbers of cells other than CD68⁺ macrophages, particularly CD3⁺ T cells and EG2⁺ eosinophils in the BAL fluid of the asthmatic subjects. When ISH was performed alone, it was shown that the asthmatic patients also had increased percentages of BAL cells expressing mRNA encoding IL-4 ($P < 0.001$), IL-5 ($P < 0.001$), and IL-2 ($P < 0.02$) when compared with the nonasthmatic controls. The percentages of cells containing IFN- γ mRNA were not significantly different (Figure 1B).

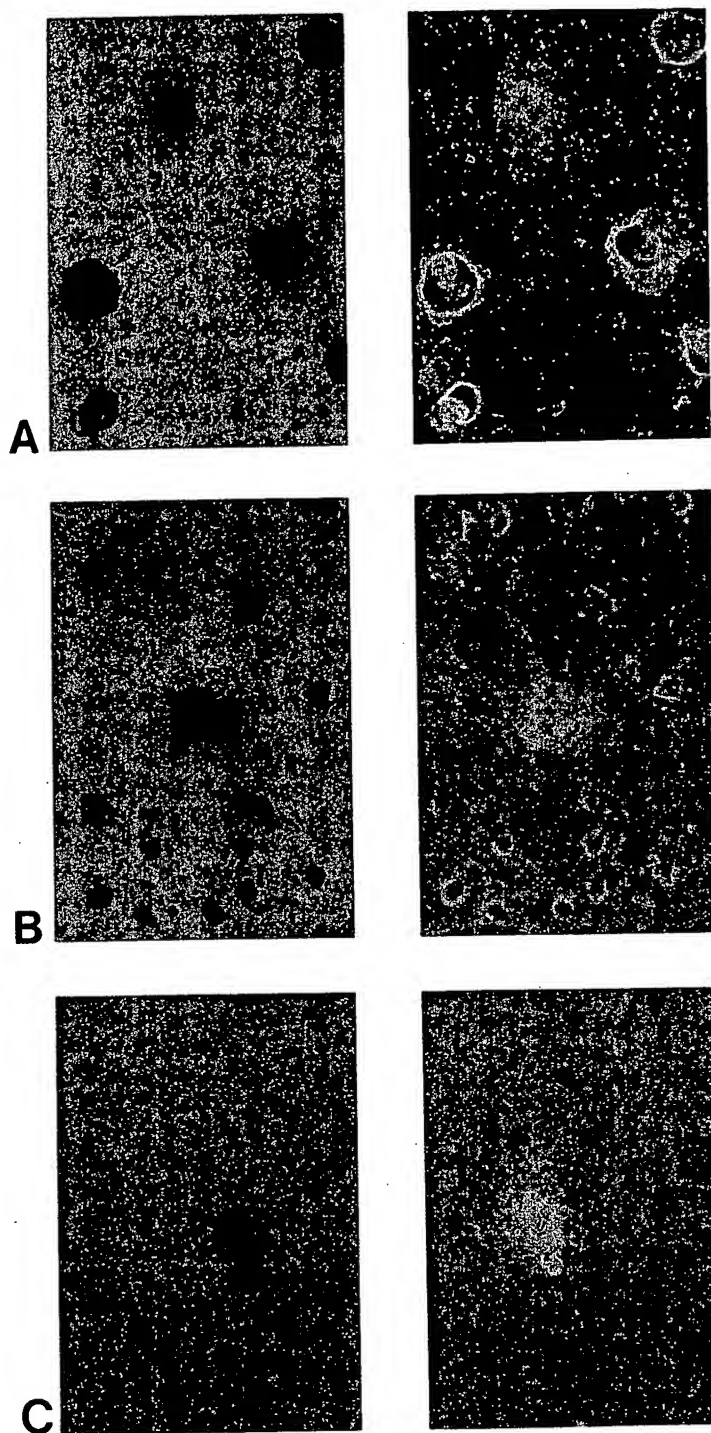


Figure 2. (A through C) Simultaneous ICC and ISH with BAL cells using the APAAP method and a ^{35}S -labeled IL-5 riboprobe. (A) Double ICC/ISH with anti-CD68 (red-staining cells) and the IL-5 riboprobe (single cell with black silver grains) shows no localization (*left*) compared with dark field (*right*). (B) In the center (*left*) there is a small cluster of double-positive ($\text{CD3}^+/\text{IL-5 mRNA}^+$) cells that by dark field (*right*) reveal two nucleated cells. (C) Colocalization of the IL-5 mRNA riboprobe to an EG2^+ eosinophil.

To phenotype the cells expressing cytokine mRNA, a combination of ICC and radioactive ISH using ^{35}S -labeled riboprobes was employed. Double-positive cells exhibited both a red color (ICC) and overlying silver grains (ISH) (Figures 2A to C). In BAL cells from the asthmatic patients, the majority of cells expressing both IL-4 and IL-5 mRNA were CD3^+ T cells ($78.5 \pm 1.8\%$ and $69.4 \pm 1.2\%$, respectively) (Figure 3). Smaller percentages of both IL-4 and IL-5 mRNA-expressing cells were accounted for by EG2^+ ($2.3 \pm 1.1\%$ and $16.3 \pm 2.6\%$) and tryptase $^+$ ($11.9 \pm$

1.7% and $9.8 \pm 1.3\%$) cells, but CD68^+ cells showed no hybridization with both probes. A few IL-4 (7.5%) and IL-5 mRNA $^+$ (4.5%) -expressing cells did not colocalize with either CD3, CD68, EG2, or tryptase-positive cells. Only occasional IL-4 and IL-5 mRNA $^+$ cells were observed in controls, and the majority of these (> 90%) colocalized with CD3^+ cells. Similarly, the low numbers of IL-2 mRNA hybridization signals observed in BAL in both asthmatic and control subjects colocalized exclusively with CD3^+ cells. Again, in both asthmatic and control subjects, the majority

% of mRNA+ cells in BAL co-expressing CD3, CD68, EG2 or tryptase

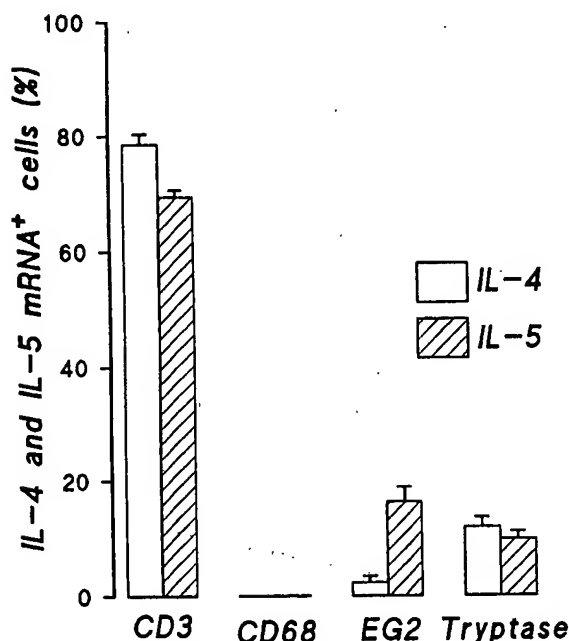


Figure 3. The percentages of IL-4 and IL-5 mRNA-positive cells in BAL from atopic asthmatic patients ($n = 7$) that coexpress CD3, CD68, EG2, and tryptase immunoreactivity.

of IFN- γ mRNA+ ($> 90\%$) cells were CD3+ cells, although occasional IFN- γ mRNA+ signals colocalized with CD68+ cells (8.4%).

When the data from the asthmatic patients were examined in terms of the percentages of cells of a given phenotype expressing IL-4 and IL-5 mRNA, it was found that 10.8% (± 1.8) and 7.5% (± 1.03) of CD3+ cells and 23.3% (± 4.7) and 13.6% (± 2.1) tryptase+ cells expressed mRNA encoding IL-4 and IL-5, respectively. Small percentages of the eo-

sophils expressed signals for both IL-4 and IL-5 mRNA ($1.5 \pm 0.54\%$ and $4.8 \pm 0.97\%$, respectively), whereas CD68+ cells, as mentioned earlier, were uniformly negative.

Bronchial Biopsies

The numbers of CD3, CD68, EG2, and tryptase-positive cells in BX from the asthmatic patients and nonasthmatic controls are compared in Figure 4A. The asthmatic patients had significantly increased numbers of EG2+ eosinophils, but not CD3+ T cells, CD68+ macrophages, or tryptase+ mast cells in BX, as compared with the controls. The majority of immunostained cells were located in the submucosa; only a minority were located within the epithelium.

In situ hybridization using digoxigenin-labeled riboprobes showed that cytokine mRNA+ cells were situated below the basement membrane, extending as far down as the smooth muscle layer (Figure 5). Comparing the asthmatic patients with the nonasthmatic controls, significant increases were observed in the numbers of cells expressing IL-4 ($27.3 \pm 4.9/\text{mm}^2$) and IL-5 mRNA ($71.9 \pm 12.9/\text{mm}^2$) (Figure 4B). There was a small but significant difference between the two groups in the numbers of cells expressing mRNA encoding IFN- γ . Unlike BAL, there was no significant difference between the two groups in mRNA expression for IL-2 (Figure 4B).

To colocalize cytokine mRNA with cytoplasmic or surface markers on the same cell, simultaneous ICC and non-radioactive ISH were employed. As mentioned previously, ICC was performed using the APAAP technique developed with a red substrate (Fast Red TR). After inactivation of the APAAP complex by paraformaldehyde fixation, the cells were hybridized with riboprobes containing a digoxigenin-AP conjugate, which was then developed using a substrate with a distinct color (NBT/BCIP, blue black). Thus, double-stained positive cells exhibited a dark blue-red color (Figures 5A through D). This mixed-color reaction has also been used simultaneously to detect distinct mRNA for bombesin-gastrin-releasing peptide and its receptor in rat brain (34). In bronchial sections, we used non-radioisotope (digoxigenin)-labeled riboprobes instead of ^{35}S -labeled probes for ISH. Although this label is not as sensitive as ra-

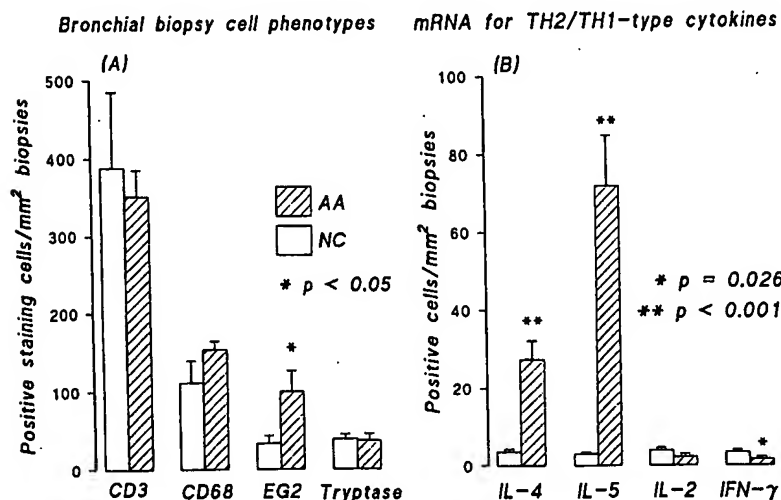


Figure 4. A comparison of BX from atopic asthmatic patients (AA) and normal nonasthmatic controls (NC) using either immunocytochemistry alone (A) for phenotypic markers or *in situ* hybridization alone (B) for cytokine mRNA+ cells. AA, $n = 7$. NC, $n = 9$.

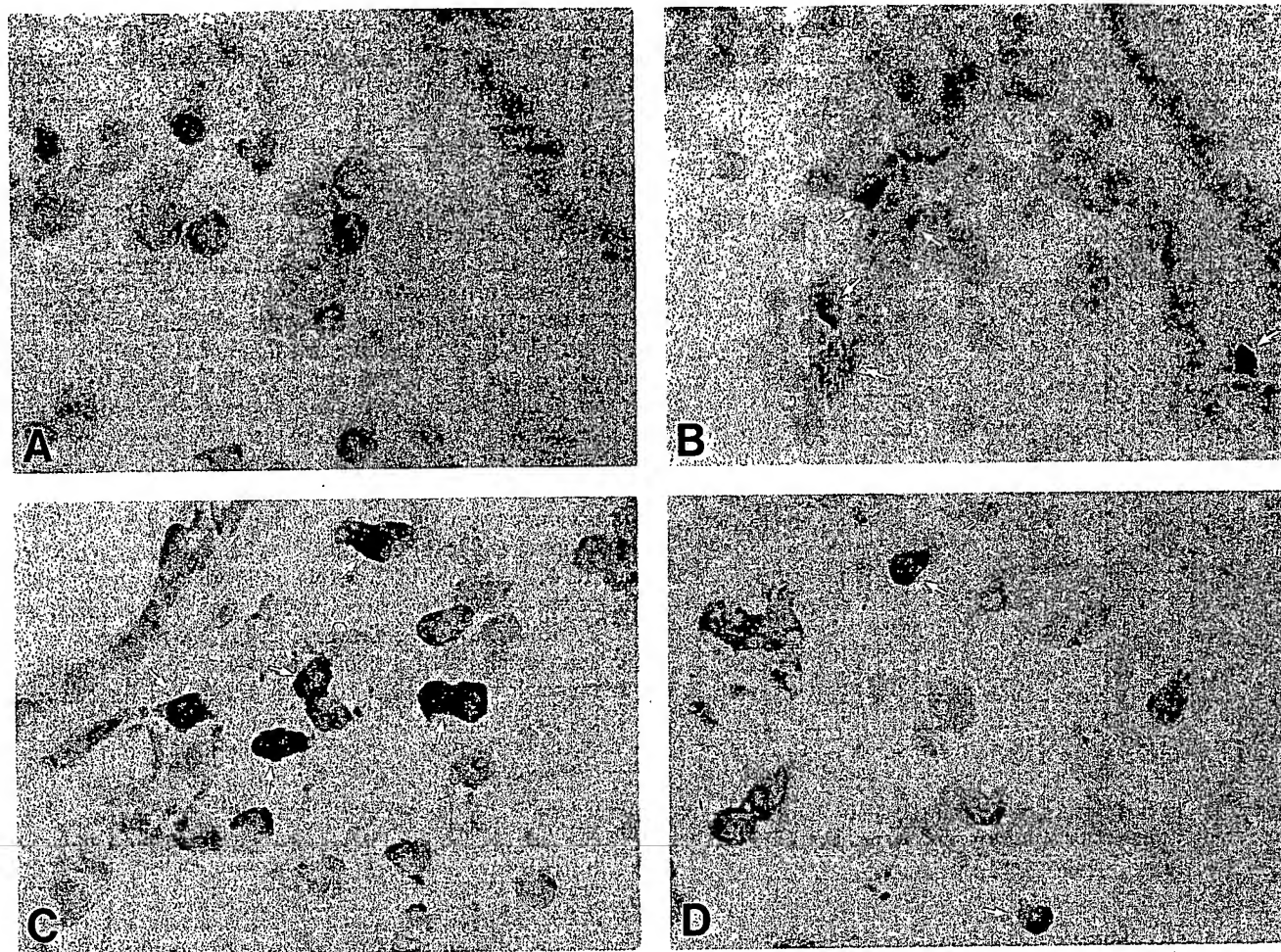


Figure 5. (A through D) Cryostat sections of a BX from a subject with atopic asthma. (A) Immunostaining alone with anti-CD3 (positive cells stained red). (B) *In situ* hybridization of a section from the same biopsy using a digoxigenin-labeled IL-5 antisense probe. Several cells (arrows) showed positive ISH signals (dark blue). (C) Simultaneous ICC and ISH of a section from the same biopsy showing CD3⁺/IL-5 mRNA⁺ double-stained (dark red) cells (arrows). (D) Double-stained CD3⁺/IL-4 mRNA⁺ cells (arrows).

dioisotope labels, a high single-cell resolution can be obtained, particularly for tissue sections (35). However, digoxigenin-labeled probes caused unacceptable background in BAL cytopsin preparations. This high background seemed to be largely the result of dust or carbon particles endocytosed by macrophages, which also have a black color similar to the blue black of NBT precipitate. Similar to BAL cells, in the atopic asthmatics, the majority of cells expressing both IL-4 and IL-5 mRNA were CD3⁺ T cells ($74.9 \pm 2.3\%$ and $73.3 \pm 2.9\%$, respectively). Smaller percentages of both IL-4 and IL-5 mRNA-expressing cells were accounted for by EG2⁺ eosinophils ($4.6 \pm 1.7\%$ and $14.1 \pm 2.3\%$) and tryptase⁺ mast cells ($14.9 \pm 1.1\%$ and $9.3 \pm 1.1\%$), but CD68⁺ macrophages showed no hybridization with both probes (Figure 6). In contrast to IL-4 and IL-5 mRNA expression, only a few cells expressing IL-2 and IFN- γ mRNA were observed in biopsies from both atopic asthmatic patients and nonasthmatic controls. Most of these IL-2 and IFN- γ mRNA signals colocalized with CD3⁺ T cells. As with BAL, a small percentage of IL-4 (5.5%) and IL-5

(3.3%) mRNA⁺ cells did not colocalize with the cells identified.

The data were then examined in terms of the percentages of cells of a given phenotype expressing IL-4 and IL-5 mRNA; the results for IL-4 were $5.9\% (\pm 0.83)$ CD3⁺/IL-4⁺, $16.97\% (\pm 4.3)$ tryptase⁺/IL-4⁺, and $1.7\% (\pm 1.09)$ EG2⁺/IL-4⁺ in atopic asthmatic patients, and $1.02\% (\pm 0.25)$ CD3⁺/IL-4⁺, $2.3\% (\pm 1.7)$ tryptase⁺/IL-4⁺, and $0.17\% (\pm 0.17)$ EG2⁺/IL-4⁺ in normal controls (Table 3). The percentages of CD3⁺, EG2⁺, and tryptase⁺ cells that coexpressed IL-5 mRNA were $15.7 \pm 3.3\%$ versus $0.99 \pm 0.22\%$, $17.4 \pm 7.7\%$ versus $0.99 \pm 0.46\%$, and $25.2 \pm 6.9\%$ versus $0.47 \pm 0.3\%$, respectively, in atopic asthmatic patients and normal controls (Table 3). Thus, there were significant increases in the numbers of CD3⁺, EG2⁺, and tryptase⁺ cells expressing TH2-type (IL-4 and IL-5 mRNA) but not TH1-type (IL-2 and IFN- γ mRNA). In contrast, the percentages of CD3⁺/IFN- γ ⁺ cells in the mucosa of atopic asthmatic patients were significantly lower than those of nonasthmatic controls ($P = 0.0172$) (Table 3).

% of mRNA⁺ cells in BX co-expressing CD3, CD68, EG2 or tryptase

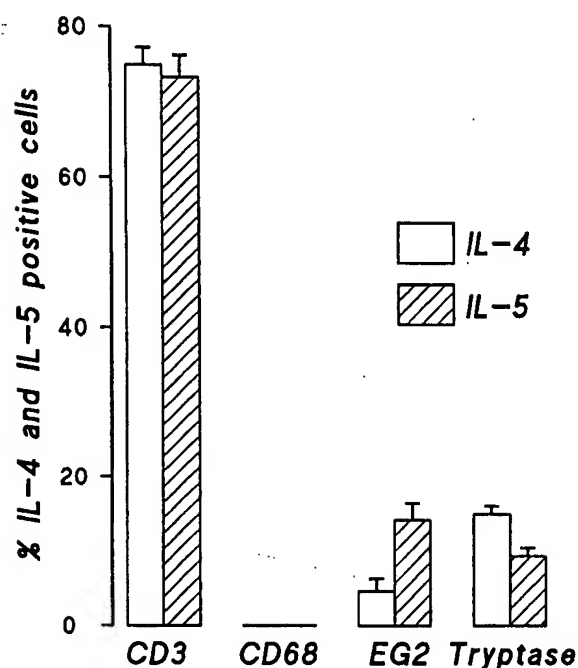


Figure 6. The percentages of IL-4 and IL-5 mRNA⁺ cells in BX from atopic asthmatic patients ($n = 7$) that coexpress CD3, CD68, EG2, and tryptase immunoreactivity.

When cell counts (ICC, ISH, and double ICC/ISH) were performed along a 1,000- μ m basement membrane to a depth of 0.45 mm, the results were virtually identical to those obtained by counting the cells per square millimeter (data not shown).

Table 4 summarizes the colocalization of IL-4 and IL-5 mRNA-expressing cells with those expressing the CD3, EG2, and tryptase phenotypic markers in BAL fluid and BX cells from the atopic asthmatic patients.

The percentages of cells of each phenotype coexpressing IL-4, IL-5, IL-2, and IFN- γ mRNA, and the absolute num-

bers of double-positive cells per square millimeter in BX from atopic asthmatic and control subjects are shown in Tables 3 and 5, respectively.

Discussion

We have previously demonstrated that increased percentages of BAL fluid cells express mRNA encoding TH2-type cytokines (IL-4 and IL-5) in atopic asthmatic patients as compared with normal controls. Enrichment of the CD2⁺ T-cell population using immunomagnetic beads confirmed that the majority of those cells expressing IL-4 and IL-5 mRNA were indeed T cells (25). To confirm these findings and to further characterize the phenotypes of cells expressing mRNA encoding these cytokines in BX and BAL fluid cells, we used a combination of ICC and ISH. This technique has been previously described (31-33). When compared with control subjects, there were increases in the percentages of EG2⁺ eosinophils and in the percentages of cells expressing mRNA for IL-4, IL-5, and IL-2 mRNA in BAL from atopic asthmatic patients. The increased percentages of cells expressing IL-2 mRNA may reflect the presence of TH0-type cells, consistent with our previous findings (25). However, no significant difference in expression of IL-2 was observed in BX between atopic asthmatic patients and normal controls. These differences may reflect local environmental influences on the cytokine expression (36). The small but significant decrease in percentages of CD68⁺ macrophages in the BAL fluid from the asthmatic patients (Figure 1A) reflects the increased absolute numbers of CD3⁺ T cells and eosinophils as described previously.

In BAL from atopic asthmatic subjects, the majority of cells expressing IL-4 and IL-5 mRNA were phenotypically CD3⁺ T lymphocytes. A relatively small percentage of EG2⁺ and tryptase⁺ cells also expressed these cytokines, in line with previous demonstrations of IL-4 mRNA and protein in mast cells (26, 27) and IL-5 mRNA expression in eosinophils (37). Our findings of IL-4 mRNA expression by a small percentage of EG2⁺ eosinophils in BAL fluid (Figure 3) and in BX (Figure 6) from atopic asthmatic subjects is novel, and has been confirmed in our own laboratory in preliminary experiments using reverse transcription (RT)-polymerase chain reaction. It is yet to be ascertained whether IL-4 transcripts can

TABLE 3

Percentages of CD3⁺, CD68⁺, EG2⁺, and tryptase⁺ cells coexpressing IL-4, IL-5, IL-2, and IFN- γ mRNA in BX*

| | CD3 ⁺ | CD68 ⁺ | EG2 ⁺ | Tryptase ⁺ |
|-------------------------------|---------------------------------|-------------------|--------------------------------|-------------------------------|
| AA IL-4 ⁺ | 5.9 \pm 0.8 $P = 0.001$ | 0 | 1.7 \pm 1.1 | 16.9 \pm 4.3 $P = 0.043$ |
| NC IL-4 ⁺ | 1.02 \pm 0.3 | 0 | 0.17 \pm 0.17 | 2.3 \pm 1.7 |
| AA IL-5 ⁺ | 15.7 \pm 3.3 $P = 0.0015$ | 0 | 17.4 \pm 7.7 $P = 0.0021$ | 25.2 \pm 6.9 $P = 0.001$ |
| NC IL-5 ⁺ | 0.99 \pm 0.22 | 0 | 0.99 \pm 0.46 | 0.47 \pm 0.3 |
| AA IL-2 ⁺ | 0.54 \pm 0.15 | 0 | 0 | 0 |
| NC IL-2 ⁺ | 0.74 \pm 0.19 | 0 | 0 | 0 |
| AA IFN- γ ⁺ | 0.33 \pm 0.11 $P = 0.0172$ | 0.04 \pm 0.04 | 0 | 0 |
| NC IFN- γ ⁺ | 0.93 \pm 0.19 | 0.3 \pm 0.18 | 0 | 0 |

* AA, atopic asthmatic patients ($n = 7$); NC, normal controls ($n = 9$).

TABLE 4
Percentages of IL-4 and IL-5 mRNA⁺ cells coexpressing CD3, EG2, and tryptase immunoreactivity in BAL and BX from atopic asthmatic patients*

| Subject | IL-4/CD3 | IL-4/EG2 | IL-4/Tryptase | IL-5/CD3 | IL-5/EG2 | IL-5/Tryptase |
|---------|----------|----------|---------------|----------|----------|---------------|
| 1 | | | | | | |
| BAL | 69.5 | 0 | 14.3 | 66.7 | 10.0 | 5.3 |
| BX | 70.0 | 9.0 | 17.0 | 60.0 | 22.9 | 11.7 |
| 2 | | | | | | |
| BAL | 77.8 | 0 | 15.0 | 71.4 | 6.7 | 12.5 |
| BX | 67.7 | 0 | 16.9 | 70.0 | 16.9 | 13.3 |
| 3 | | | | | | |
| BAL | 80.0 | 6.7 | 14.8 | 70.3 | 12.5 | 8.3 |
| BX | 71.4 | 10.0 | 10.0 | 78.7 | 11.9 | 10.3 |
| 4 | | | | | | |
| BAL | 85.7 | 0 | 7.7 | 65.0 | 22.2 | 16.7 |
| BX | 81.5 | 0 | 16.2 | 67.6 | 20.0 | 10.7 |
| 5 | | | | | | |
| BAL | 79.2 | 4.7 | 16.1 | 66.7 | 21.1 | 5.9 |
| BX | 83.8 | 0 | 17.6 | 77.6 | 10.2 | 5.9 |
| 6 | | | | | | |
| BAL | 77.8 | 0 | 11.3 | 74.1 | 16.7 | 12.5 |
| BX | 73.8 | 5.4 | 15.6 | 78.3 | 10.0 | 6.0 |
| 7 | | | | | | |
| BAL | 79.2 | 4.5 | 4.3 | 71.4 | 25.0 | 14.3 |
| BX | 76.2 | 7.7 | 11.4 | 80.8 | 6.6 | 7.1 |

* No localization of IL-4 or IL-5 mRNA to CD68⁺ cells was observed.

TABLE 5
Number of double-positive cells per square millimeter of biopsies*

| | CD3 ⁺ | CD68 ⁺ | EG2 ⁺ | Tryptase ⁺ |
|-----------------------|--------------------------|-------------------|-------------------------|--------------------------|
| AA IL-4 ⁺ | 20.4 ± 3.4 P = 0.001 | 0 | 1.5 ± 0.76 | 4.6 ± 0.63 P = 0.0015 |
| NC IL-4 ⁺ | 2.8 ± 0.43 | 0 | 0.078 ± 0.078 | 0.67 ± 0.37 |
| AA IL-5 ⁺ | 51.8 ± 8.5 P = 0.001 | 0 | 10.9 ± 3.5 P = 0.001 | 7.4 ± 1.9 P = 0.001 |
| NC IL-5 ⁺ | 2.3 ± 0.28 | 0 | 0.5 ± 0.2 | 0.13 ± 0.09 |
| AA IL-2 ⁺ | 1.9 ± 0.58 | 0 | 0 | 0 |
| NC IL-2 ⁺ | 2.9 ± 0.9 | 0 | 0 | 0 |
| AA IFN-γ ⁺ | 1.2 ± 0.44 P = 0.0262 | 0.07 ± 0.07 | 0 | 0 |
| NC IFN-γ ⁺ | 2.8 ± 0.4 | 0.23 ± 0.12 | 0 | 0 |

* AA, atopic asthmatic patients (n = 7); NC, normal controls (n = 9).

be induced in eosinophils, *in vivo* and *in vitro*, by various specific and nonimmunologic stimuli.

Interestingly, in the asthmatic patients a few IL-4 and IL-5 mRNA-positive signals in BAL fluid cells (7.3 and 4.5%, respectively) and in BX (5.5 and 3.3%) did not colocalize with any of the cells identified by ICC (i.e., CD3⁺, EG2⁺, tryptase⁺, and CD68⁺). This suggests that cells other than T cells, eosinophils, mast cells, and macrophages have the capacity to secrete IL-4 and IL-5 in asthmatic patients, at least at the mRNA level. We used parallel bronchial sections and counted all positive cells. However, it was impossible to detect all cell phenotypes expressing distinct cytokine mRNA on the same section, because only one cellular marker and one cytokine mRNA could be detected simultaneously on each section (6 μm). In contrast, in normal controls, all cytokine mRNA signals colocalized with CD3⁺ T cells, although the amount of expression of these signals was greatly reduced as

compared with the asthmatic patients. Nevertheless, T cells seemed to be the principal source of mRNA, at least for IL-4 and IL-5. It would be interesting to determine whether the T cells expressing cytokine mRNA were activated (i.e., CD25⁺) and allergen specific.

In contrast to TH2-like cytokine mRNA, all of the IL-2 mRNA signals colocalized with CD3⁺ cells in BAL from both asthmatic patients and normal controls. In BAL from atopic asthmatic patients, IFN-γ mRNA mostly colocalized with CD3⁺ cells. In the controls a small number of IFN-γ mRNA signals colocalized with CD68⁺ cells. This has been previously described (33, 38).

To validate ISH using the nonradioactive antidigoxigenin-alkaline phosphatase conjugate, we first established that several cells in the biopsies exhibited strong dark blue staining with the IL-4 and IL-5 riboprobes (Figure 5). The specificity of these hybridization signals was confirmed by the

absence of staining with either the sense probes, after RNase treatment, or unrelated probes. Furthermore, there was no signal when the antidigoxigenin-alkaline phosphatase conjugate was omitted in the developing stage. Using the same method, we demonstrated that T cells were the principal source of IL-4 and IL-5 mRNA in biopsies from allergen-induced rhinitis (31, 32).

As with BAL, mast cells in biopsies also expressed mRNA for IL-4 and IL-5, even though their numbers were 3-fold fewer than the numbers of T cells (Figure 6). Using immunostaining, both IL-4 and IL-5 products have been detected in mast cells in BX (27), suggesting that mast cells are also a potential source of TH2-type cytokines.

Interestingly, there was a significant decrease in CD3⁺ IFN- γ ⁺ cells in the atopic asthmatic subjects compared with the controls (Tables 3 and 5), which may account for the observed reduction of IFN- γ mRNA expression in the asthmatic subjects (Figure 4B). It has been suggested that the balance of local IL-4 and IFN- γ secretion may regulate the synthesis of IgE (39). Increased expression of IFN- γ mRNA has been observed in atopic asthmatic patients after treatment with prednisolone (40) and in atopic rhinitic patients after immunotherapy (41). Thus, this relative reduction of IFN- γ expression may contribute to the elevated serum concentration of IgE, which is a feature of atopic asthma. A recent study using an animal model showed that CD8⁺ cells inhibited IgE production and normalized airway responsiveness by release of IFN- γ (42). Thus, it would be informative in further studies to identify whether IFN- γ ⁺ cells are CD4⁺, CD8⁺, or neither.

Two of the nonasthmatic controls in our studies were atopic, as defined by positive skin-prick testing, although neither had bronchial hyperresponsiveness (Table 1). Although it is theoretically possible that atopic status *per se* might have had some influence on cytokine mRNA expression by inflammatory cells in the bronchial mucosa and BAL fluid of these nonasthmatic subjects, neither of these subjects suffered from atopic disease and only one was likely to have been exposed to allergens, to which he was sensitized (control subject 2, house dust mite) at the time of the study. For these reasons we feel justified in including these subjects in our analysis, although clearly an investigation of how cytokine expression may be influenced by atopic status is of extreme importance, and is indeed the subject of ongoing further investigation in our laboratory.

Studies such as this are likely to represent "snapshots" of what is likely a highly dynamic process in which the contribution of various cells to local cytokine production may vary with time and with the clinical setting of the disease. Clearly, longitudinal studies and detailed comparisons of the molecular immunopathology of asthma in diverse clinical settings (such as mild versus severe asthma, treated versus untreated asthma, occupational asthma, and allergen-exacerbated asthma) are required in the future. We have already published data showing that BAL fluid T cells remain the principal source of TH2-type cytokines, at the mRNA level, following allergen bronchial challenge of atopic asthmatic patients (43), and that glucocorticoid therapy of mild atopic asthmatic patients was accompanied by a reduction in the expression of mRNA encoding TH2-type cytokines (40), the principal source of which we have demonstrated to be T cells.

We have no corresponding information as yet concerning the bronchial mucosa. Although expression of mRNA does not necessarily equate with release of the corresponding protein, a recent study (44) demonstrated the presence of TH2-type cytokine protein in CD4⁺ T cells in BX obtained from atopic asthmatic patients. Given, however, the recent clear demonstrations in this and other studies of mast cells (27) and eosinophils (28) as potential sources of IL-4 and IL-5, a more complete evaluation of the role of cytokine synthesis by these cells, which may contribute to autocrine eosinophil accumulation and activation, as well as steering of the T-cell response forward to TH2-type pattern, is necessary in a wide variety of disease settings.

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PREDOMINANT T_{H2}-LIKE BRONCHOALVEOLAR T-LYMPHOCYTE POPULATION IN ATOPIC ASTHMA

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Abstract Background. In atopic asthma, activated T helper lymphocytes are present in bronchial-biopsy specimens and bronchoalveolar-lavage (BAL) fluid, and their production of cytokines may be important in the pathogenesis of this disorder. Different patterns of cytokine release are characteristic of certain subgroups of T helper cells, termed T_{H1} and T_{H2}, the former mediating delayed-type hypersensitivity and the latter mediating IgE synthesis and eosinophilia. The pattern of cytokine production in atopic asthma is unknown.

Methods. We assessed cells obtained by BAL in subjects with mild atopic asthma and in normal control subjects for the expression of messenger RNA (mRNA) for interleukin-2, 3, 4, and 5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma by in situ hybridization with ³²P-labeled complementary RNA. Localization of mRNA to BAL T cells was assessed by

simultaneous in situ hybridization and immunofluorescence and by in situ hybridization after immunomagnetic enrichment or depletion of T cells.

Results. As compared with the control subjects, the subjects with asthma had more BAL cells per 1000 cells that were positive for mRNA for interleukin-2 (P<0.05), 3 (P<0.01), 4 (P<0.001), and 5 (P<0.001) and GM-CSF (P<0.001). There was no significant difference between the two groups in the number of cells expressing mRNA for interferon gamma. In the subjects with asthma, mRNA for interleukin-4 and 5 was expressed predominantly by T lymphocytes.

Conclusions. Atopic asthma is associated with activation in the bronchi of the interleukin-3, 4, and 5 and GM-CSF gene cluster, a pattern compatible with predominant activation of the T_{H2}-like T-cell population. (N Engl J Med 1992;326:298-304.)

BRONCHIAL inflammation in asthma may depend in part on the activation of T helper lymphocytes that elaborate proinflammatory cytokines. CD4-positive lymphocytes in peripheral blood from patients with severe acute asthma demonstrate increased expression of activation markers as compared with those from patients with chronic airflow limitation or normal controls. These changes resolve with improvement of lung function.¹ Activation of peripheral-blood T cells also occurs in patients with milder asthma.² The number of activated T cells correlates with the degree of peripheral-blood eosinophilia. On electron microscopy, bronchial-biopsy specimens from patients with asthma reveal atypical lymphocyte morphologic features suggestive of activation,³ and on immunocytochemical analysis an increased number of cells stain for the T-cell-activation marker CD25 (the interleukin-2 receptor).⁴ Flow-cytometric analysis of cells in bronchoalveolar-lavage (BAL) fluid from patients with asthma also reveals increased expression of CD25 by CD4-positive lymphocytes. The degree of activation of CD4-positive lymphocytes correlates with the number of eosinophils in BAL fluid, as well as the severity of symptoms and the degree of bronchial hyperresponsiveness.⁵

Studies of T-cell clones support the hypothesis that activation of a subgroup of helper cells leads to the release of cytokines important in the immunopathologic processes of allergic disease. When murine T-cell clones are divided on the basis of expression of messenger RNA (mRNA) for, and their release of, cytokines,⁶ T_{H1} cells produce interleukin-2 and interferon gamma, but no interleukin-4 or 5, whereas T_{H2} clones produce interleukin-4 and 5 but no interleukin-2 or interferon gamma.⁶ Both produce interleukin-3

and granulocyte-macrophage colony-stimulating factor (GM-CSF). T_{H1} clones mediate delayed-type hypersensitivity, whereas T_{H2} clones promote preferential B-cell production of IgE, IgA, IgG1, and IgM.^{7,8} Interferon gamma from T_{H1} cells inhibits the proliferation of T_{H2} clones.⁹ T_{H2} cells produce interleukin-10, which inhibits the production of cytokine by T_{H1} cells.¹⁰ Thus, one or the other pattern of cytokine production might be expected to predominate in a T-cell response, and the predominant pattern may determine the type of inflammatory response.¹¹

Since interleukin-5 promotes the differentiation,¹² vascular adhesion,¹³ and in vitro survival¹⁴ of eosinophils as well as enhances histamine release from basophils,¹⁵ and since interleukin-4 is a mast-cell growth factor¹⁶ and also promotes the switching of B-cell isotypes to the production of IgE,¹⁷ T_{H2}-like T cells may be particularly implicated in allergic disease. This possibility is supported by recent evidence demonstrating the preferential release of T_{H2}-like cytokines by allergen-specific T-cell clones from donors with atopic disease^{18,19} and also by a recent study demonstrating a T_{H2}-like pattern of cytokine mRNA expression in the allergen-induced late cutaneous response.²⁰

The aim of the present study was to examine the profile of cytokine mRNA expression in cells obtained by BAL in subjects with atopic asthma and control subjects. The hypothesis tested was that T cells in BAL fluid from subjects with atopic asthma express mRNA for a T_{H2} pattern of cytokines and that this pattern of cytokine release determines at least in part the nature of the inflammatory response seen in the bronchial mucosa in mild asthma.

METHODS

Patients

The clinical and demographic characteristics of the subjects with asthma and the normal controls are shown in Table 1. Subjects with asthma were included on the basis of history and a demonstrated

Table 1. Clinical and Demographic Characteristics of the Study Subjects.

| STUDY SUBJECTS | AGE | SEX | ALLERGEN* | | | FEV ₁ | METHACHOLINE PC ₂₀ | SYMPTOMS |
|-----------------------|------------|-------|-----------------|-----------------------|-----|------------------|-------------------------------|----------|
| | | | GRASS POLLEN | HOUSE DUST MITE | CAT | | | |
| | | | | | | % | mg/ml | |
| Subjects with asthma† | | | | | | | | |
| 1 | 26 | F | - | + | + | 90 | 0.4 | Yes |
| 2 | 25 | F | - | + | + | 85 | 0.7 | Yes |
| 3 | 24 | M | - | + | + | 75 | 0.8 | Yes |
| 4 | 22 | F | + | + | + | 82 | 0.3 | Yes |
| 5 | 25 | F | + | + | - | 108 | 0.4 | Yes |
| 6 | 23 | F | + | + | - | 81 | 0.3 | Yes |
| 7 | 27 | F | + | + | - | 87 | 0.9 | Yes |
| 8 | 28 | F | + | - | - | 100 | 4.6 | No |
| 9 | 23 | M | + | - | - | 96 | 19.9 | No |
| 10 | 28 | M | + | - | - | 98 | 15.5 | No |
| 11 | 22 | F | + | - | - | 79 | 3.2 | Yes |
| 12 | 19 | F | + | - | - | 96 | 13.8 | No |
| 13 | 24 | F | + | + | + | 97 | 1.0 | Yes |
| 14 | 19 | M | + | + | - | 87 | 16.0 | No |
| 15 | 32 | M | + | + | + | 75 | 4.0 | Yes |
| Controls (n = 10)‡ | 25 (18-33) | 6F/4M | - | - | - | 103 (95-118) | All >32 | No |

*Allergen sensitivity was determined by skin-prick test and radioallergosorbent test. Plus signs indicate positive results, and minus signs negative results.

†The subjects with asthma were asymptomatic subjects with seasonal asthma who were studied out of season. BAL cells from Subjects 11 through 15 were used for immunomagnetic isolation of T cells and dual analysis.

‡Values shown for age and FEV₁ are means, with ranges given in parentheses.

reversible airflow limitation (20 percent variability in forced expiratory volume in one second [FEV₁] or peak expiratory flow rate), increased airway responsiveness to methacholine (concentration producing a decrease of 20 percent from base line in FEV₁ [PC₂₀], <8 mg per milliliter), or both. The subjects with asthma were atopic, as defined by positive skin-prick tests and radioallergosorbent tests (Phadebas, Pharmacia, Uppsala, Sweden) for common aeroallergens. All the study subjects were nonsmokers; none had received corticosteroid therapy in the three months before the study began. Informed consent was obtained from all the subjects. The study was approved by the ethics committee of the Royal Brompton National Heart and Lung Hospital. The subjects with asthma were studied without allergen challenge, since the aim of the study was to determine the cytokine pattern involved in base-line inflammatory changes. The normal controls had no history of allergic disease, negative skin-prick and radioallergosorbent tests, normal FEV₁, and a PC₂₀ of more than 32 mg per milliliter.

Fiberoptic Bronchoscopy

Bronchoscopy was performed by the same operator in both the subjects with asthma and the control subjects after they had received 2.5 mg of albuterol by nebulizer, 0.6 mg of atropine, midazolam for sedation, and 2 percent or 4 percent lidocaine for local anesthesia. BAL was performed by instilling four 60-ml aliquots of warmed, pH-adjusted, normal saline into either the right middle lobe or the lingula.

Processing of BAL Cells

Care was taken at all times to avoid contamination of the BAL samples with RNase. BAL cells were centrifuged at 300×g for seven minutes, washed once in RPMI 1640 medium with HEPES buffer (Flow Laboratories, Irvine, Scotland), and resuspended in 1.5 ml of RPMI 1640 medium with 0.5 percent bovine serum albumin (Sigma Chemicals, Poole, United Kingdom) and 0.1 percent sodium azide. After being counted on a Neubauer hemocytometer, cells were resuspended at a concentration of 1×10⁵ cells per milliliter in RPMI 1640 and cytopsin slides were made with a Shandon 2 cytopsin device (Shandon Southern Instruments, Runcorn, United Kingdom). For cell differentiation, slides were stained with May-Grünwald-Giemsa, and mast cells were enumerated with alcian blue and safranin. For in situ hybridization, glass slides were washed in Decon detergent (Decon Laboratories, Hove, United Kingdom) and then

alcohol and baked at 200°C before they were coated with poly-L-lysine (molecular weight, >150,000; Sigma). Samples prepared on these slides were air-dried for 5 minutes, then fixed in 4 percent paraformaldehyde in phosphate-buffered saline (pH 7.2; BDH, Poole, United Kingdom) for 30 minutes, followed by two changes of 15 percent phosphate-buffered saline-sucrose for 1 hour each. Slides were incubated at 37°C, then stored at -80°C before hybridization.

In Situ Hybridization

Chemicals and reagents for in situ hybridization were supplied by Sigma unless otherwise stated. Radiolabeled RNA probes were prepared as previously described.²¹ In brief, complementary DNA (cDNA) was inserted into the appropriate vector and linearized to produce antisense (complementary sequence to cytokine mRNA) and sense (identical sequence to cytokine mRNA) probes. Labeled transcripts were synthesized in the presence of adenosine triphosphate, guanosine triphosphate, cytidine triphosphate, and ³²P-labeled uridine triphosphate (Amersham International, Amersham, United Kingdom) and SP6 or T7 polymerases (Promega Biotechnology, Southampton, United Kingdom) to generate antisense or sense probes, respectively.

BAL cytopsin slides were rehydrated, then cells were made permeable with Triton X-100 and proteinase K. Cells were then fixed in 4 percent paraformaldehyde to terminate proteinase K activity. After washing in phosphate-buffered saline, slides were immersed in 0.25 percent acetic anhydride and 0.1 mol of triethanolamine per liter, then prehybridized in 50 percent formamide and 2× saline-sodium citrate buffer (SSC) (1× SSC was 0.15 mol of sodium chloride per liter, 0.15 mol of sodium citrate per liter, pH 7; BDH). Nonspecific binding of cRNA probes to eosinophils has been described,²² but we avoided this in our preparations through the use of reducing agents. For hybridization, 10 µl of hybridization buffer containing 1×10⁶ cpm of labeled RNA probe was applied to each slide. Slides were then covered and incubated at 42°C for 16 hours. After hybridization the slides were washed in decreasing concentrations of SSC (from 4× to 0.1×) at 42°C. Unhybridized RNA probe was removed with 10 µg of RNase A per milliliter in 2× SSC (15 minutes at 37°C), and the slides were then dehydrated with alcohols containing 0.3 percent ammonium acetate before air-drying. Autoradiography was performed by immersing the slides in emulsion (K-5, Ilford, Moberley, United Kingdom) and exposing them for three to five days before development (D-19, Kodak, Hemel Hempstead, United Kingdom).

Hybrids between cytokine mRNA and cRNA probes were localized as dense collections of silver grains overlying cells. Since it was not possible to count individual silver grains, we quantified cells expressing cytokine mRNA in terms of the number of cells with overlying silver grains per 1000 cells in the cytopsin preparation. At least two slides were hybridized for each probe, and counts were performed in triplicate. The mean coefficient of variation for cell counts was 5 percent. Negative controls were cytopsin slides hybridized with sense probe and slides pretreated with RNase before hybridization with antisense probe. Positive controls were cytopsin slides made from a concanavalin A-stimulated human T-cell clone known to produce interleukin-3, 4, and 5 and GM-CSF, or from phytohemagglutinin-stimulated mononuclear cells for interleukin-2 and interferon gamma.

Simultaneous Identification of CD3 and Cytokine mRNA

To examine whether mRNA for interleukin-4 and 5 was expressed by T cells, cytocentrifuge preparations of BAL cells from three subjects with asthma were fixed in 4 percent paraformaldehyde and 15 percent sucrose in phosphate-buffered saline. Cells were incubated simultaneously with interleukin-4 or 5 cRNA probes labeled with uridine triphosphate-biotin (Universal Biological,

London) and with monoclonal antibody to CD3 directly conjugated to fluorescein isothiocyanate (Becton Dickinson, Cowley, United Kingdom). The conditions for in situ hybridization were as previously described,²³ and positive hybridization of probe and cytokine mRNA was detected with use of streptavidin-Texas red staining (Amersham). Controls were IgG1-fluorescein isothiocyanate with sense probes or RNase pretreatment. Cells were quantified by fluorescence microscopy, and the percentage of cells expressing both CD3 and cytokine mRNA as well as the percentage of singly labeled cells was evaluated by counting at least 200 positive cells.

Immunomagnetic Cell Separation

To examine further whether T cells were the source of interleukin-4 and 5 mRNA, BAL cells from five subjects with asthma were incubated with immunomagnetic beads (Dynabeads, Dynal, Wirral, United Kingdom) covalently bound to monoclonal antibody to CD2 in a ratio of three beads to one lymphocyte for 20 minutes at 4°C. Cells were separated with a magnetic cell separator (Dynal²⁴). CD2-positive cells were washed four times in phosphate-buffered saline with 0.1 percent bovine serum albumin, and cytospin preparations were made from both positively and negatively separated cells as well as from unseparated cells for differential counts and in situ hybridization for interleukin-4 and 5 mRNA. In situ hybridization was measured in terms of the number of cells with overlying silver grains as a percentage of the total number of cells on cytocentrifuge preparations of BAL cells in positively or negatively separated and unseparated samples. These results were compared with the percentages of lymphocytes on cytospin slides as determined on May-Grünwald-Giemsa staining.

Statistical Analysis

Statistical comparison of BAL differential counts and in situ hybridization results was performed with the Mann-Whitney U test with Minitab PC software (Minitab, State College, Pa.).

RESULTS

May-Grünwald-Giemsa staining of cytocentrifuge preparations showed a higher percentage of eosinophils in BAL fluid from the subjects with asthma (median, 3.6 percent) than in fluid from the controls (median, 0.4 percent; 95 percent confidence interval for the difference, 0.7 percent to 5.5 percent; $P < 0.002$). A small but significant increase in the percentage of alcian blue-positive mast cells was also observed in BAL fluid from the subjects with asthma (0.3 percent vs. 0 percent; 95 percent confidence interval for the difference, 0.1 percent to 0.3 percent; $P < 0.002$). The total number of cells and the percentages of other cell types (alveolar macrophages, lymphocytes, neutrophils, and epithelial cells) did not differ between the two groups.

Positive in situ hybridization signals for cytokine mRNA were observed as dense collections of silver grains overlying BAL cells on autoradiographs only when antisense probes were used (Fig. 1 through 6). There were no signals from BAL cytospin slides hybridized with sense probes, nor from slides pretreated with RNase. There was a strong positive signal from positive-control cytospin slides. A variable number of positive hybridization signals was observed for each cytokine in both the subjects with asthma and the controls. Cytokine mRNA for interleukin-2, 3, 4, and 5 appeared to be localized in smaller cells, whereas in situ hybridization for GM-CSF mRNA resulted in silver grains over both large and small cells (Fig. 5).

Figure 7 shows the number of cells per 1000 in BAL cytospin preparations expressing mRNA for cytokines. There were increased numbers of cells expressing mRNA for interleukin-2, 3, 4, and 5 and GM-CSF in BAL cytospin preparations from the subjects with asthma as compared with preparations from the controls. In contrast, there was no difference in the number of cells expressing mRNA for interferon gamma.

Dual fluorescence for CD3 and mRNA for interleukin-4 or 5 in BAL cytospin preparations from three subjects with asthma showed that a mean of 91 percent of the cells positive for interleukin-5 mRNA were positive for CD3 (89 percent, 92 percent, and 91 percent in the three subjects), and 58 percent of the CD3-positive cells expressed interleukin-5 mRNA. Ninety-two percent of the cells expressing mRNA for interleukin-4 were positive for CD3 (97 percent, 91 percent, and 88 percent), and 73 percent of the CD3-positive cells expressed interleukin-4 mRNA.

The results of cell counts and in situ hybridization for mRNA for interleukin-4 and 5 in BAL cytocentrifuge preparations after immunomagnetic enrichment or depletion of CD2-positive cells (T-lymphocytes), together with the results in unseparated BAL cells, are shown in Table 2 for five subjects with asthma. The clear association between cytokine mRNA and CD2-positive cells was further confirmed by the association of anti-CD2-coated beads and mRNA-positive cells in cytospin preparations, which identified these cells as T lymphocytes (Fig. 8).

DISCUSSION

The observation of increased numbers of cells expressing mRNA for interleukin-2, 3, 4, and 5 and GM-CSF, but not interferon gamma, together with the localization of interleukin-4 and 5 mRNA to T lymphocytes in BAL fluid from subjects with atopic asthma, provides evidence of a T_H2 -like pattern of cytokine-gene expression in allergic asthma. The presence of an increased number of cells expressing sufficient copy numbers of mRNA for cytokines to be detected by in situ hybridization is at least compatible with the notion of increased cytokine production, although this remains to be confirmed.

The method of counting cells with a positive hybridization signal for mRNA that we used here is semiquantitative, giving an estimate of the proportion of cells within the sample that transcribe cytokine genes, but not the number of copies of mRNA per cell. Nonetheless, this method does allow a comparative analysis, and it demonstrated a marked difference between subjects with asthma and control subjects. The limited number of cells recovered by BAL precluded cell separation and simultaneous immunohistochemical analysis and in situ hybridization in the subjects whose BAL cells were used for in situ hybridization for the full range of cytokines, but we have no reason to suppose that the results would not be comparable.

Since the initial description of two subtypes of murine T-cell clones classified according to cytokine pro-

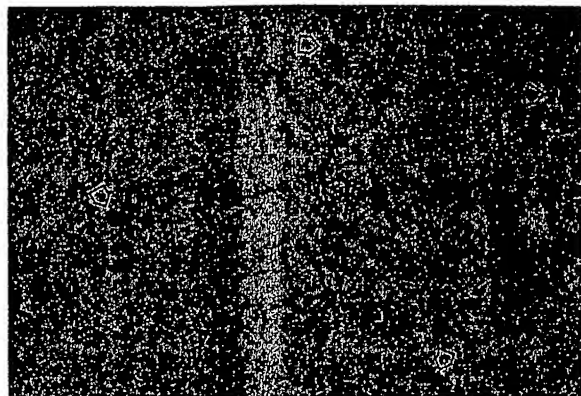


Figure 1. Autoradiograph Showing Signals from BAL Cells from a Subject with Asthma Hybridized with an Antisense Riboprobe to Interleukin-5.

Positive signals appear as dense collections of silver grains overlying cells (arrows).

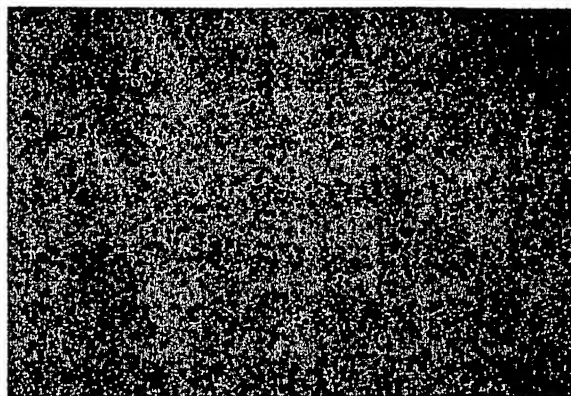


Figure 2. Autoradiograph of BAL Cells from a Subject with Asthma Hybridized with a Sense Riboprobe to Interleukin-5.

No hybridization signal is seen.

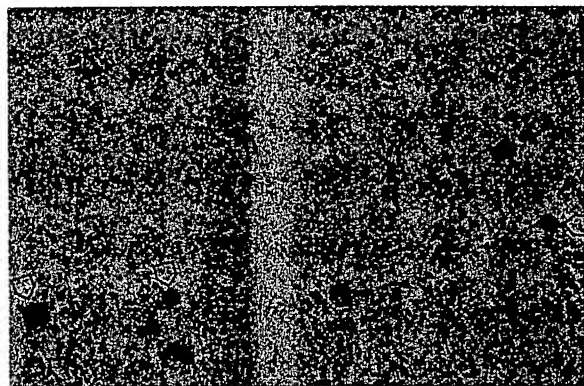


Figure 3. Autoradiograph Showing Signals from BAL Cells from a Subject with Asthma Hybridized with an Antisense Riboprobe to Interleukin-4.

Positive signals appear as dense collections of silver grains overlying cells (arrows).

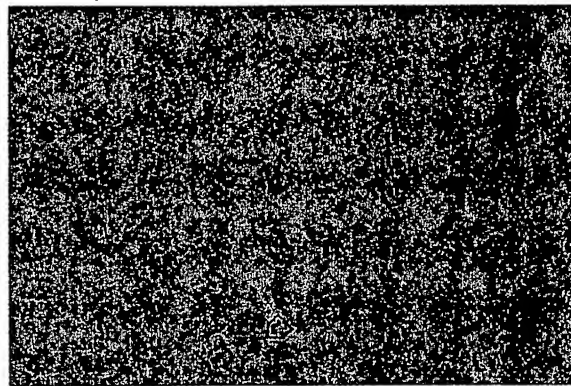


Figure 4. Autoradiograph Showing Signals from BAL Cells from a Control Subject Hybridized with an Antisense Riboprobe to Interleukin-4.

A few BAL cells give a positive hybridization signal for interleukin-4 (arrow).

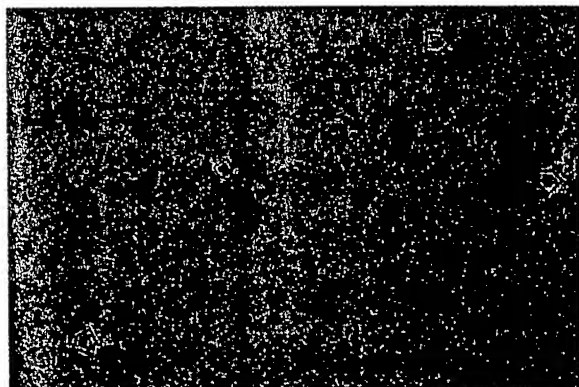


Figure 5. Autoradiograph Showing Signals from BAL Cells from a Subject with Asthma Hybridized with an Antisense Riboprobe to GM-CSF.

Positive signals appear as dense collections of silver grains overlying cells (arrows).

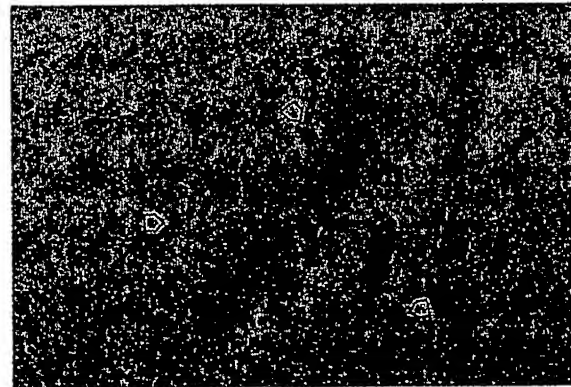


Figure 6. Autoradiograph Showing Signals from BAL Cells from a Subject with Asthma Hybridized with an Antisense Riboprobe to Interleukin-3.

Positive signals appear as dense collections of silver grains overlying cells (arrows).

duction, the possibility that the T_{H2} pattern is relevant to allergic disease has been apparent.^{25,26} We have previously described evidence of the activation of CD4-positive T lymphocytes in asthma,^{1,4,5} and the present results suggest that activated T helper cells in mild atopic asthma may be a human T_{H2} equivalent. The isolation of T cells from BAL fluid for clonal analysis will be required for further confirmation, since it is not possible to determine from the present study whether the various cytokines were synthesized by the same T cells.

It would be of interest to explore the cytokine profile of BAL T cells in other lung diseases, particularly those characterized by granulomatous inflammation in which a T_{H1} pattern might be expected. The spontaneous production of both interleukin-2 and interferon gamma from BAL T cells has been reported in patients with active sarcoidosis^{27,28} although the full pattern of cytokine production remains to be elucidated.

Although the double-immunofluorescence and CD2-enhancement studies strongly suggest that the majority of cytokine mRNA originated from T cells, other cells in the BAL fluid may in theory have been responsible for a proportion of the cytokine mRNA observed. The synthesis of interleukin-3, 4, and 5 and GM-CSF by murine mast-cell lines was recently described.^{29,30} However, mast cells constituted not more than 1 percent of the cells in the BAL cytospin preparations in this study. It has been shown that alveolar macrophages from patients with asthma can produce GM-CSF in response to allergen,³¹ and the GM-CSF mRNA observed in our subjects may have been pro-

Table 2. Association of Interleukin-4 and 5 mRNA Signals with CD2-Positive Cells from Five Subjects with Asthma.*

| EXPERIMENT NO. | % POSITIVE FOR CYTOKINE mRNA | | | % LYMPHOCYTES† | | |
|----------------|------------------------------|------|----|----------------|------|----|
| | CD2+ | CD2- | NS | CD2+ | CD2- | NS |
| Interleukin-4 | | | | | | |
| 1 | 40 | 1 | 8 | 85 | 3 | 13 |
| 2 | 42 | 2 | 7 | 65 | 4 | 11 |
| 3 | 57 | 2 | 7 | 70 | 4 | 14 |
| 4 | 62 | 0 | 4 | 62 | 1 | 14 |
| 5 | 39 | 1 | 8 | 73 | 3 | 8 |
| Median | 42 | 1 | 7 | 70 | 3 | 13 |
| Interleukin-5 | | | | | | |
| 1 | 52 | 1 | 7 | 85 | 3 | 13 |
| 2 | 37 | 3 | 8 | 65 | 4 | 11 |
| 3 | 62 | 2 | 7 | 70 | 4 | 14 |
| 4 | 70 | 4 | 11 | 62 | 1 | 14 |
| 5 | 59 | 2 | 6 | 73 | 3 | 8 |
| Median | 59 | 2 | 7 | 70 | 3 | 13 |

*Values are shown for cytospin preparations of BAL cells separated by immunomagnetic beads coupled to anti-CD2 (CD2+), negatively selected cells (CD2-), and cells not subjected to separation (NS).

†On May-Grünwald-Giemsa staining.

duced in part by alveolar macrophages (Fig. 5). Eosinophils have been shown to be capable of producing interleukin-1,³² transforming growth factor- α ,³³ and GM-CSF.³⁴

The pattern of cytokine mRNA expression by BAL cells from subjects with atopic asthma differs from the murine T_{H2} pattern⁶ and the mRNA profile described in allergen-induced late cutaneous responses.²⁰ In this situation there were no significant differences in the number of cells positive for interleukin-2 mRNA between skin-biopsy specimens from sites challenged with allergen and those from sites challenged with diluent. It is also of note that BAL cells from control subjects showed some positive signals for cytokine mRNA and that, in contrast with the skin-biopsy findings, mRNA for interferon gamma was present in a small number of cells in BAL fluid from both groups. Although an increased number of BAL cells were positive for interleukin-2 mRNA in the subjects with asthma, the difference was not as marked as for interleukin-3, 4, and 5 and GM-CSF. The interleukin-2 mRNA may represent a minority population of activated T_{H1} -equivalent cells, as was found in T-cell clones isolated from mice infected with *Nippostrongylus brasiliensis*, in which the majority of T-helper clones were of the T_{H2} pattern,¹¹ or it may represent a T_{H0} pattern from some newly activated cells. Alternatively, this may be the human pattern of T_{H2} cytokines, as described by Wierenga et al. in T-cell clones from donors with atopic disease.¹⁸ The majority of

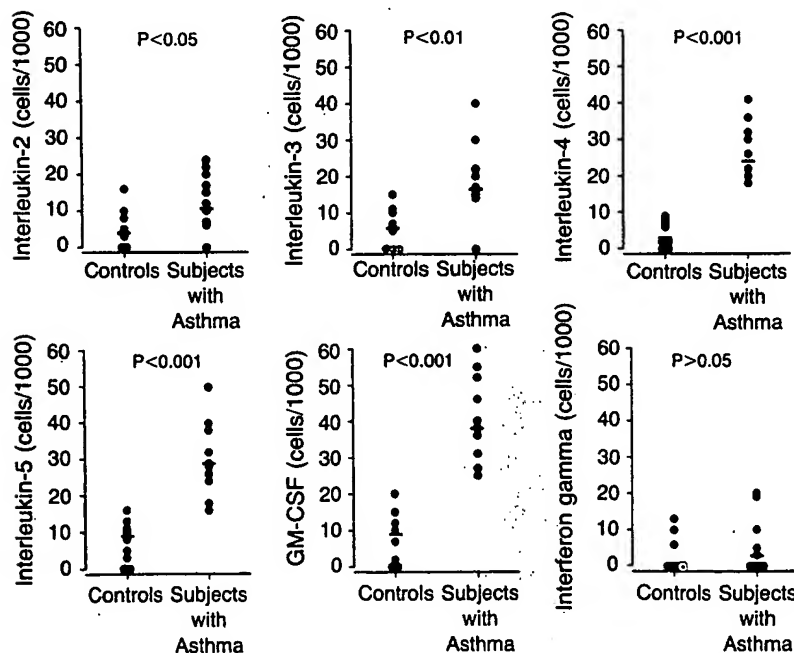


Figure 7. Number of Cells with Positive Signals for mRNA for Interleukin-2, 3, 4, and 5, GM-CSF, and Interferon Gamma in BAL Fluid from 10 Subjects with Asthma and 10 Control Subjects.

Significantly more cells were positive for interleukin-2, 3, 4, and 5 and GM-CSF mRNA, but not for interferon gamma mRNA, in the subjects with asthma. Values were compared by the Mann-Whitney U test. Bars indicate median values.

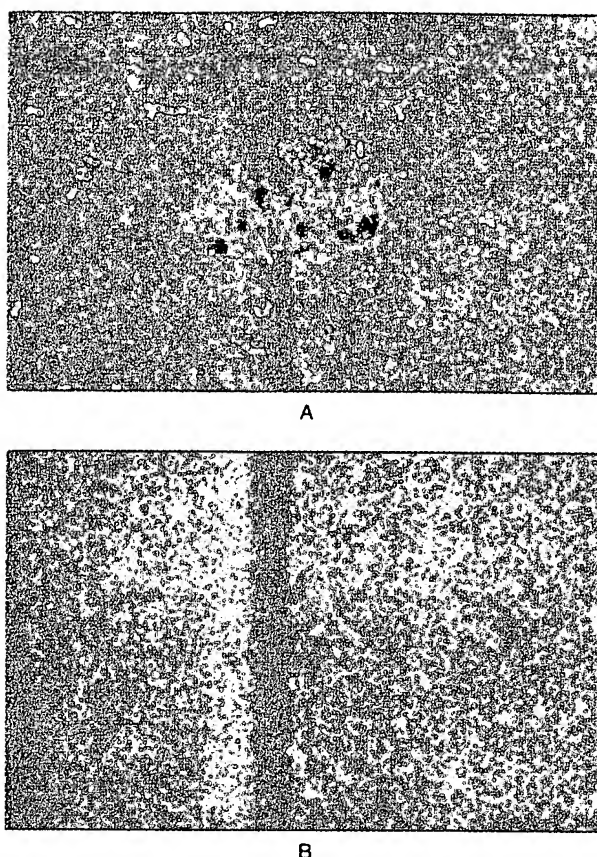


Figure 8. Autoradiographs of Cytospin Preparations of BAL Cells from Subjects with Asthma after in Situ Hybridization with Interleukin-5 Antisense Probes and Immunomagnetic Sorting with CD2-Linked Beads.

In Panel A, a positive hybridization signal for interleukin-5 mRNA is seen in association with CD2-positive cells in a positively selected sample. In Panel B, no cells positive for interleukin-5 mRNA are seen in the sample negatively selected to exclude CD2-positive cells.

T helper cells in BAL fluid were of the CD45RO memory phenotype (data not shown), and it has been suggested that these cells produce interleukin-2 because of cross-reactive stimulation.³⁵

The mechanisms determining the selection of the T_{H2} phenotype in atopic disease are of considerable importance to our further understanding of allergy. In both individual mice and humans, there appears to be an antigen-dependent selection of cytokine patterns. The local cytokine milieu may have an important role in selecting the subgroup that predominates in an inflammatory response: interleukin-4 appears to favor T_{H2} development,³⁶ whereas interferon gamma favors T_{H1}.⁹ There appear to be differences between T_{H1} and T_{H2} murine T-cell clones in terms of both post-receptor signaling³⁷ and preferred antigen-presenting cells.³⁸ Whether this results from a requirement for different costimulatory signals or reflects an innate difference in the mechanism of T-cell activation remains to be elucidated. These differences may provide an exciting opportunity for therapeutic manipulation of different T-helper subgroups, with possible applications in allergy and other diseases.

We are indebted to Dr. Andrew Wardlaw and Ms. Angela Rance for helping with probe preparation, Qiu Meng for expert assistance with in situ hybridization, Dr. D. Quint for supplying the T-cell clone used as a positive control, Dr. C. Sanderson for supplying interleukin-5 cDNA, and Glaxo Biogen, Geneva, for supplying cDNA for interleukin-2, 3, and 4, GM-CSF, and interferon gamma.

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Oxazolone Colitis: A Murine Model of T Helper Cell Type 2 Colitis Treatable with Antibodies to Interleukin 4

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Summary

In this study we describe oxazolone colitis, a new form of experimental colitis. This model is induced in SJL/J mice by the rectal instillation of the haptenating agent, oxazolone, and is characterized by a rapidly developing colitis confined to the distal half of the colon; it consists of a mixed neutrophil/lymphocyte infiltration limited to the superficial layer of the mucosa which is associated with ulceration. Oxazolone colitis is a T helper cell type 2 (Th2)-mediated process since stimulated T cells from lesional tissue produce markedly increased amounts of interleukin (IL)-4 and IL-5; in addition, anti-IL-4 administration leads to a striking amelioration of disease, whereas anti-IL-12 administration either has no effect or exacerbates disease. Finally, this proinflammatory Th2 cytokine response is counterbalanced by a massive transforming growth factor- β (TGF- β) response which limits both the extent and duration of disease: lesional (distal) T cells manifest a 20–30-fold increase in TGF- β production, whereas nonlesional (proximal) T cells manifest an even greater 40–50-fold increase. In addition, anti-TGF- β administration leads to more severe inflammation which now involves the entire colon. The histologic features and distribution of oxazolone colitis have characteristics that resemble ulcerative colitis (UC) and thus sharply distinguish this model from most other models, which usually resemble Crohn's disease. This feature of oxazolone colitis as well as its cytokine profile have important implications to the pathogenesis and treatment of UC.

Key words: hapten • inflammation • cytokine • T helper cell type 2 • transforming growth factor- β

Hapten-induced experimental colitis in mice (i.e., TNBS¹ colitis induced by the haptenating agent, 2,4,6-trinitrobenzene sulfonic acid) has proven to be an exceptionally useful model of certain forms of human inflammatory bowel disease. For example, the study of this model has led to the recognition that an IL-12-driven, Th1 T cell-mediated inflammation of the colon is not only prevented by the systemic administration of anti-IL-12 antibody, but can also be treated by such administration (1). This observation has provided the theoretical justification for the use of inhibitors of IL-12, including anti-IL-12 itself, in the treatment of Crohn's disease, an inflammation also dominated by a Th1 T cell response (2–5). Studies of the TNBS colitis model have also shown that administration of TNBS per rectum and per os have very different effects; rectal administration results in severe colitis whereas oral administration (either in the form of haptenated co-

lionic protein or TNBS itself) leads to the induction of suppressor T cells producing TGF- β and the inhibition of colitis caused by TNBS given simultaneously by the rectal route (6, 7). These findings in concert with similar findings in other models establish that mucosal inflammation and/or its prevention depend at least in part on a balance between proinflammatory Th1 T cell responses and antiinflammatory TGF- β responses (1, 6–10).

While, as indicated above, TNBS has proven a useful agent in the induction of experimental colitis, its effects on the colon may be limited by the range of T cell responses it is capable of inducing. In this regard, previous studies imply that haptenating agents differ somewhat with respect to the cell populations they address and thus differ somewhat in the type of immune responses they induce (11–16). On this basis, it seemed possible that administration of other haptenating agents per rectum to mice might elicit a different type of colitis. An additional reason for exploring the colitogenic potential of a second haptenating agent arises from the fact that the feeding of a haptenating agent, as alluded to above, results in antigen nonspecific suppressor T cell responses which could potentially mediate bystander suppres-

¹ Abbreviations used in this paper: LP, lamina propria; TNBS, trinitrobenzene sulfonic acid; UC, ulcerative colitis.

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sion of a colitis induced by an unrelated haptenating reagent (6, 7, 17–23). Thus, the identification of a second colitogenic haptenating reagent would allow one to test the possibility that the feeding of a haptenating agent could nonspecifically suppress (treat) colitis occurring in humans.

In this study we explored these possibilities by studying the colitogenic potential of the “classical” haptenating agent, oxazolone (24, 25). We found that oxazolone at the dose administered elicited a very different colitis than that obtained with TNBS administration in that it induced a colitis involving only the distal half of the colon and had histologic features resembling ulcerative colitis (UC) rather than Crohn’s disease. In addition, oxazolone colitis is IL-4-driven rather than IL-12-driven, is prevented by the administration of anti-IL-4, and is exacerbated by the administration of anti-IL-12. Finally, we found that per rectal administration of oxazolone, in contrast to TNBS, induces a TGF- β response which plays an important role in limiting the inflammation both in extent and in time.

Materials and Methods

Induction of Colitis. Colitis was studied in specific pathogen-free, 5–6-wk-old male SJL/J mice obtained from the National Cancer Institute (NCI, National Institutes of Health [NIH], Bethesda, MD) and maintained in the National Institute of Allergy and Infectious Diseases (NIAID) animal facility. For induction of colitis, mice were first lightly anesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL) and then 6 mg of the haptenating agent, oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) (Sigma Chemical Co., St. Louis, MO), was administered per rectum via a 3.5 F catheter equipped with a 1-ml syringe. The catheter was inserted so that the tip was 4 cm proximal to the anal verge and the oxazolone was injected with a total volume of 150 μ l of a 1:1 H₂O/ethanol mixture (50% ethanol). To ensure distribution of the oxazolone within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Control mice were administered 50% ethanol alone using the same technique.

Histologic Assessment of Colitis. Tissues obtained at indicated time points were fixed in 10% buffered formalin phosphate and then embedded in paraffin, cut into sections, and then stained with hematoxylin and eosin. Stained sections were examined for evidence of colitis using as criteria the presence of infiltration with lymphocyte, macrophages, or polymorphonuclear cells, elongation and/or distortion of crypts, crypt abscesses, reduction in goblet cell number, frank ulceration, and edema formation.

Isolation and Purification of Lamina Propria (LP) T Cells. LP T cells were isolated from freshly obtained colonic specimens using a modification of the technique described by Van der Heijden and Stok (26). Using this technique, the colonic specimens were first washed in HBSS-calcium magnesium free and cut into 0.5-cm pieces. They were then incubated twice, each time for 15 min in HBSS containing EDTA (0.37 mg/ml) and dithiothreitol (0.145 mg/ml) at 37°C. The tissue was then digested further in RPMI containing collagenase D (400 U/ml) and DNase I (0.01 mg/ml) (both obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN) in a shaking incubator at 37°C. The LP cells released from the tissue were then resuspended in 100% Percoll, layered under a 40% Percoll gradient (Pharmacia Biotech AB, Uppsala, Sweden), and spun at 1,800 rpm to obtain the lymphocyte-enriched

population accumulating at the 40–100% interface. Finally, the lymphocyte-enriched population was further purified by negative selection using an Isocell mouse T cell isolation column (Pierce Chemical Co., Rockford, IL). The resultant T cell population, when analyzed by flow cytometry, using a FACScan® (Becton Dickinson, Sunnyvale, CA), was shown to be composed of >90% CD3⁺ T cells.

Isolation and Purification of Spleen T Cells. For isolation of spleen T cells, spleens were aseptically removed. Cells were dispersed in 1 \times PBS by applied pressure to spleen tissue. The dispersed splenocytes were then filtered through a 100- μ m filter and depleted of RBCs by hypotonic lysis with ACK lysing buffer (Biofluids Inc., Rockville, MD) using a standard technique (27). The cells were then layered on a 40–100% Percoll gradient and spun at 1,800 rpm to obtain the lymphocyte-rich cells at the 40–100% interface. The cells were further purified by negative selection using a mouse T cell isolation column as described above. The resultant T cell population when analyzed by flow cytometry was shown to be composed of >90% CD3⁺ T cells.

Culture of LP T Cells and Spleen T Cells for Assay of Cytokine Production. Culture of LP T cells and spleen T cells was generally performed using complete medium consisting of RPMI 1640 (Whittaker M.A. Bioproducts, Inc., Walkersville, MD) supplemented with 3 mM L-glutamine, 10 mM Hepes buffer, 10 μ g/ml gentamycin, 100 U/ml each of penicillin and streptomycin (Whittaker M.A. Bioproducts, Inc.), 0.05 mM 2-ME, and 10% by volume FCS (Sigma Chemical Co.). When cells were cultured for evaluation of TGF- β production, serum-free media supplemented with 1% nutridoma-SP (Boehringer Mannheim Biochemicals) were used. To measure cytokine production 10⁶ LP T cells or spleen T cells purified as described above were loaded into uncoated culture wells (to measure production by unstimulated cells) or wells coated with murine anti-CD3 ϵ antibody (clone 145-2C11; PharMingen, San Diego, CA) and 1 μ g/ml soluble anti-CD28 (clone 37.51; PharMingen) (to measure production by stimulated cells) and cultured for 48 h (or 60 h in the case of TGF- β). The culture supernatants were then harvested and assayed for cytokine concentration by ELISA. The culture plates used in these studies were 24-well Costar plates (Costar Corp., Cambridge, MA); coating of these plates with anti-CD3 antibody was accomplished by exposing wells to a solution containing anti-CD3 ϵ antibody (10 μ g/ml) in carbonate buffer (pH 9.6) overnight at 4°C.

ELISA Assays. Cytokine concentrations (except for TGF- β) were determined by commercially available specific ELISA assays using duo-paired murine cytokines as per the manufacturer’s recommendations (Endogen Corp., Woburn, MA) on Immulon-4, 96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). TGF- β concentrations were determined using the Predicta TGF- β ELISA assay (Genzyme Corp., Cambridge, MA). Optical densities were measured on a Dynatech MR 5000 ELISA reader at a wavelength of 490 nm.

Treatment of Mice with Anticytokine Antibodies. Mice were administered various anticytokine antibodies via intraperitoneal injection at the time of disease induction with oxazolone. Rat anti-mouse IL-4 (3 mg per dose), murine anti-human TGF- β 1,2,3 (1 mg per dose), and rat anti-mouse IL-12 (2 mg per dose) as well as isotype control (rat IgG2a) were used. Anti-IL-4 consisted of the monoclonal antibody produced from the 11B11 hybridoma cell line donated by Dr. William Paul (Laboratory Immuno-regulation, NIAID, NIH); this antibody was purified from ascites fluid produced in nude mice by precipitation in 50% saturated ammonium sulfate and then dialyzed overnight as per protocol (28).

Anti-TGF- β consisted of the 1.D11.6 monoclonal antibody purchased in purified form from the Genzyme Corp. Anti-IL-12 consisted of the monoclonal antibody produced by the C17.8 hybridoma cell line supplied by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA); this antibody was purified from ascites fluid produced in nude mice and was purified using E-Z-SEP purification kits (Middlesex Sciences, Inc., Foxborough, MA) according to manufacturer's protocol. Control rat IgG2a was obtained from Jackson ImmunoResearch (West Grove, PA).

Results

Intrarectal Administration of Oxazolone Induces a UC-like Colonic Inflammation in SJL/J Mice. In previous studies we and others have shown that the administration of the haptenating agent TNBS induces an IL-12-driven, Th1 T cell colitis resembling Crohn's disease (1, 6, 7, 29, 30). In studies directed at exploring if similar disease is obtained with other haptenating agents, we subjected SJL/J mice to intrarectal administration of oxazolone (6 mg, dissolved in 50% ethanol), a haptenating agent that does not cross-react with TNBS (12, 13, 15, 23). As shown in Fig. 1, SJL/J mice so treated reproducibly developed a rapid onset colitis marked by weight loss and diarrhea peaking by day 2 after oxazolone administration and leading to death of 50% of the mice by day 4. Thereafter, surviving mice at days 4–7 after oxazolone administration slowly increased their weight and by days 10–12 the majority of the mice were free of diarrhea and appeared healthy.

The above clinical picture of oxazolone colitis is vastly different from TNBS colitis, a colitis with a more gradual onset that peaks much later (7 d after induction) and is more persistent. The two diseases also differ on the macro-

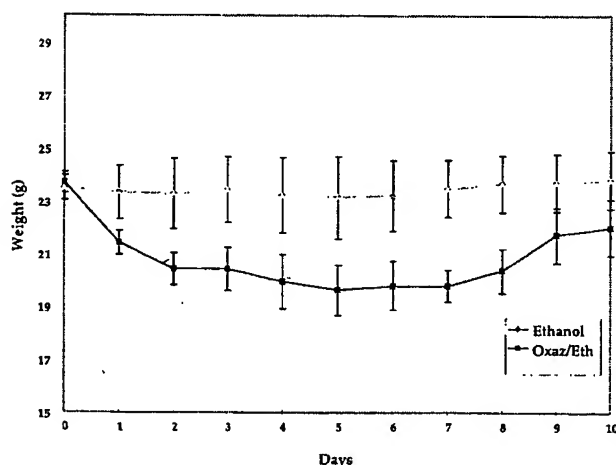


Figure 1. Wasting disease in mice with oxazolone colitis. Intrarectal administration of oxazolone induces rapid onset of severe bloody diarrhea and wasting disease. Shown are weight changes over a 10-d period occurring in normal SJL/J control mice treated with 50% ethanol alone, and SJL/J mice treated with oxazolone in 50% ethanol. Data are from one representative experiment (out of a total of three experiments). Each point represents average weight data pooled from five mice. Standard errors are indicated.

scopic and microscopic levels. Thus, as shown in Fig. 2, on macroscopic examination of oxazolone colitis at 48 h after oxazolone administration, a severe hemorrhagic colitis which remarkably involves only the distal 50% of the bowel is observed; this is in contrast to TNBS colitis in which inflammation involving the entire length of the bowel is seen. In addition, as shown in Fig. 3, on microscopic examination of involved colon of oxazolone-treated mice (again at 48 h) a superficial inflammation characterized by the presence of epithelial cell loss and patchy ulceration, pronounced depletion of mucin producing-goblet cells, and reduction of the density of the tubular glands is present. In addition, in the LP, a mixed inflammatory cell infiltrate consisting of lymphocytes and granulocytes (the latter consisting mostly of neutrophils and, to a lesser extent, eosinophils) associated with an exudation of cells into the bowel lumen is observed. Finally, the submucosal layer displays marked edema with few inflammatory cells, while in the outer muscle layer one sees little or no evidence of inflammation at all. The foregoing changes in the involved areas of the colon are continuous, but end abruptly in mid-colon and in the noninvolved colon a normal microscopic appearance, i.e., no inflammation, is seen. These various changes are in obvious contrast to those found in TNBS colitis where one observes a transmural inflammation involving all layers of the bowel wall that is not associated with the presence of cellular exudation or the presence of significant numbers of neutrophils or eosinophils.

The colons of mice killed after the inflammation had clinically subsided (at 10 d after intrarectal administration of oxazolone) showed some evidence of the earlier presence of inflammation. On microscopic analysis of the colonic tissue, one could see evidence of a resolving inflammatory process, including the presence of epithelial regeneration (mitotic figures), reappearance of goblet cells, and a residual

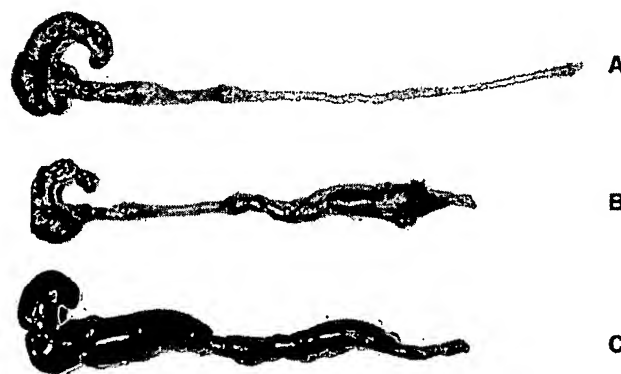


Figure 2. Macroscopic changes of colons in hapten-treated mice. Photographs of dissected large intestine of (A) normal SJL/J control mouse treated with 50% ethanol, (B) SJL/J mouse treated with oxazolone in 50% ethanol 2 d after initial rectal administration, and (C) appearance of colons of mice with TNBS-induced colitis 7 d after intrarectal administration. The colons of oxazolone-treated mice display a hemorrhagic edematous colon limited to the distal half of the colon. This is in contrast to colons in TNBS-treated mice which display an inflammation of the entire colon.

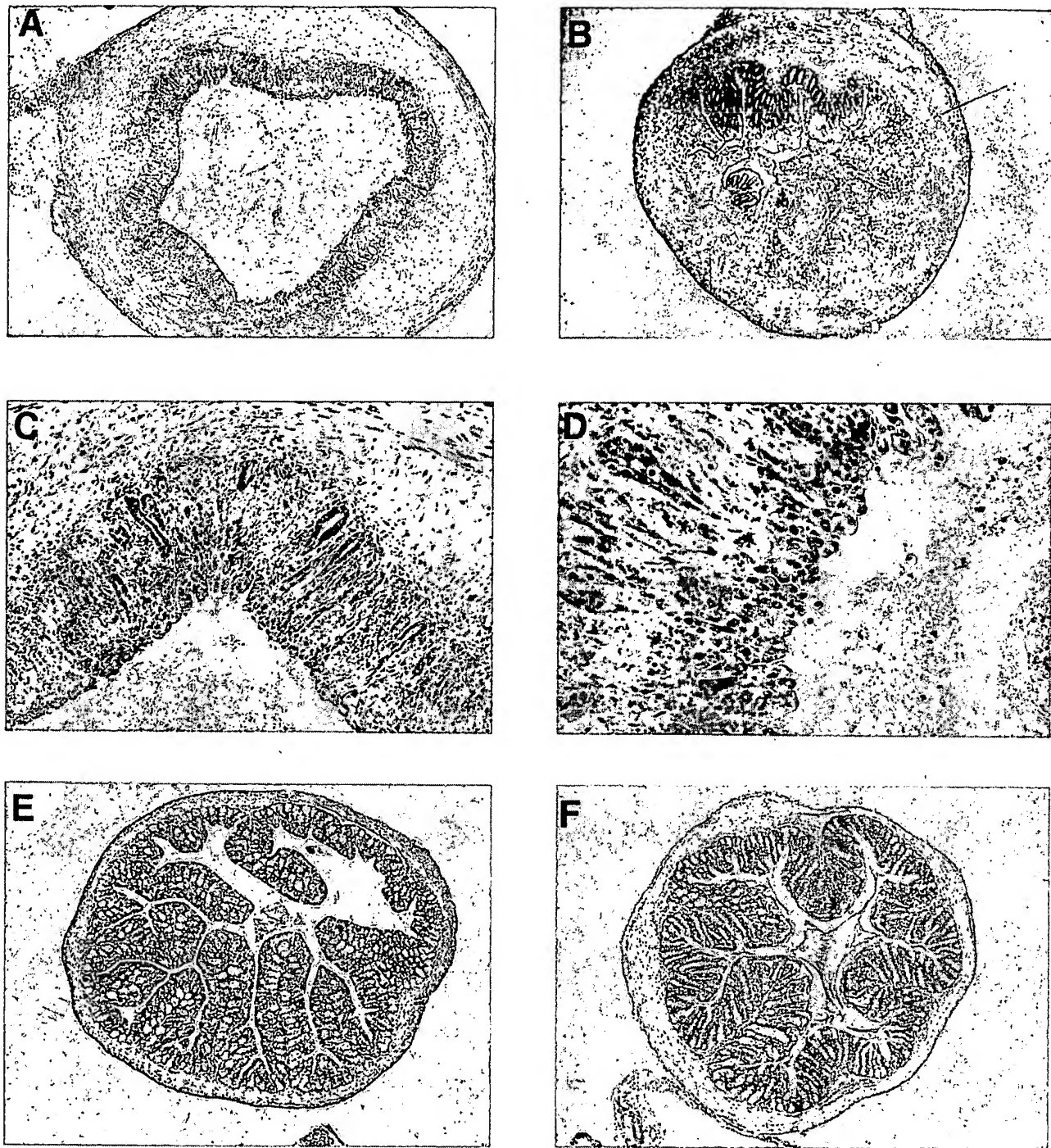


Figure 3. Histologic analysis of the colons from SJL/J mice with hapten-induced colitis and control mice. (A) Photomicrograph of hematoxylin and eosin-stained (H/E) paraffin section of distal colon ($\times 100$) from an oxazolone-treated mouse on day 2. Significant edema with inflammatory infiltrates localized to the superficial mucosal layer is present. (B) H/E-stained section of colon ($\times 100$) from a TNBS-treated mouse at 7 d after intrarectal administration: a severe transmural colitis with bowel wall thickening is seen. (C) Photomicrograph of H/E-stained section of colon ($\times 150$) from an oxazolone-treated mouse showing the presence of superficial hemorrhage, ulceration, distortion of the crypts, loss of goblet cells, and mucin depletion. (D) High-power micrograph of cross-section of H/E-stained section of colon ($\times 400$) of an oxazolone-treated mouse, showing a mixed lymphocytic infiltrate localized to the superficial layers of the mucosa and the presence of a luminal cellular exudate. (E) Photomicrograph of H/E-stained cross-section from the proximal colon ($\times 100$) of an oxazolone-treated mouse showing only a small amount of mucosal edema and lymphocytic infiltrate. (F) Photomicrograph of H/E-stained cross-section of normal control ($\times 100$) colon 2 d after ethanol administration.

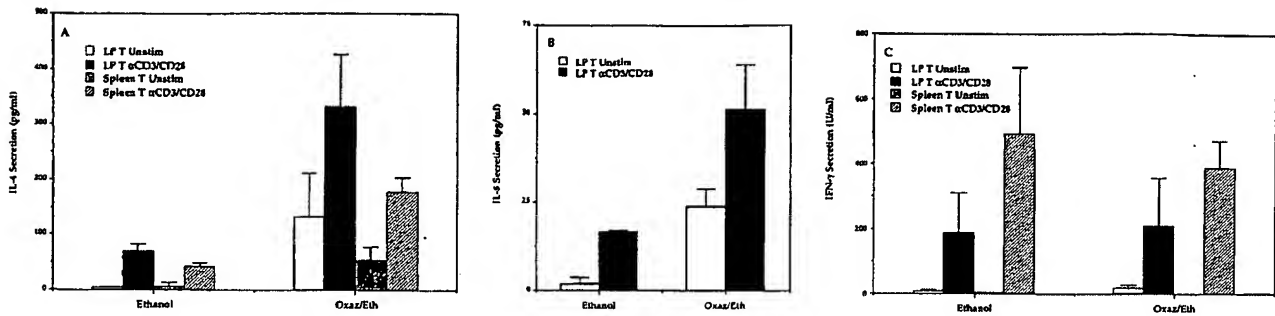


Figure 4. Cytokine production of stimulated and unstimulated LP and spleen T cells in oxazolone-induced colitis. LP T and spleen T cells were isolated from oxazolone and control (ethanol-treated) mice on day 2 and cultured for 48 h in the presence or absence of anti-CD3 and anti-CD28 (see Materials and Methods). Culture supernatants were analyzed for concentrations of IL-4, IL-5, and IFN- γ secretion by specific ELISA. Data shown represent pooled values from three independent experiments. Standard errors are indicated.

inflammatory infiltrate consisting mainly of lymphocytes, but few if any granulocytes. Again, this inflammatory cell infiltrate was confined to the superficial layer of the mucosa and did not involve the outer, muscle layer (data not shown).

Taken together, these macroscopic and microscopic histologic features of oxazolone colitis are highly reminiscent of the features of the human inflammatory bowel disease, UC, and thus differ greatly from TNBS colitis, which more closely resembles Crohn's disease.

The Colonic Inflammation Characteristic of Oxazolone Colitis Is Associated with the Presence of T Cells Having a Th2 T Cell Profile. To characterize the nature of the immune response in oxazolone colitis, we extracted T cells from inflamed LP and spleen specimens (at 48 h after oxazolone administration) and determined their capacity to secrete cytokines after stimulation with anti-CD3/anti-CD28 antibodies in vitro as described in Materials and Methods. As shown in Fig. 4 A, LP T cells (as well as spleen T cells) of mice with oxazolone colitis manifested an ~10-fold increase in spontaneous (unstimulated) IL-4 production and a 5-fold increase in anti-CD3/anti-CD28-induced IL-4 production as compared with control mice ($P < 0.01$). In addition, as shown in Fig. 4 B, mice with oxazolone colitis demonstrated a >10-fold increase in unstimulated production of IL-5 and a 3-fold increase in anti-CD3/anti-CD28-induced production of IL-5 as compared with control mice ($P < 0.05$). In contrast, as shown in Fig. 4 C, LP T cells (as well as spleen T cells) of mice with oxazolone colitis manifested no increase in unstimulated or stimulated production of IFN- γ as compared with cells from control mice ($P > 0.05$). These data thus show that oxazolone colitis is associated with the presence of LP cells that are strongly skewed toward production of Th2 cytokines.

In further studies we compared cytokine secretion exhibited by LP T cells isolated from inflamed distal colon with T cells isolated from noninflamed proximal colon. As shown in Fig. 5, whereas anti-CD3/CD28-induced T cells from inflamed areas produced increased amounts of IL-4 as shown above ($P < 0.01$), T cells from noninflamed areas produce levels of IL-4 similar to that of control T cells ($P >$

0.05). It should be noted, however, that the T cells from the uninfamed tissue were not completely normal in that they secreted an increased amount of IL-4, as compared with control colonic T cells when cultured in the absence of stimulants ($P < 0.01$).

Oxazolone-induced Colitis Is Associated with Increased Production of the Suppressor Cytokine TGF- β . In previous studies of experimental colitis associated with a Th1 T cell response (such as TNBS colitis) it was shown that the T cell response and thus the colitis were counterregulated by the presence of T cells producing TGF- β (6–10). On this basis, we measured the capacity of purified LP T cells from whole colon and spleen specimens of mice with oxazolone colitis to produce TGF- β . As shown in Fig. 6 A, T cells of such mice (either LP T cells or spleen T cells) exhibited a 15-fold increase in TGF- β production when cultured without stimulation or a 30-fold increase in TGF- β production when cultured in the presence of anti-CD3/CD28, as compared with cells from control mice ($P <$

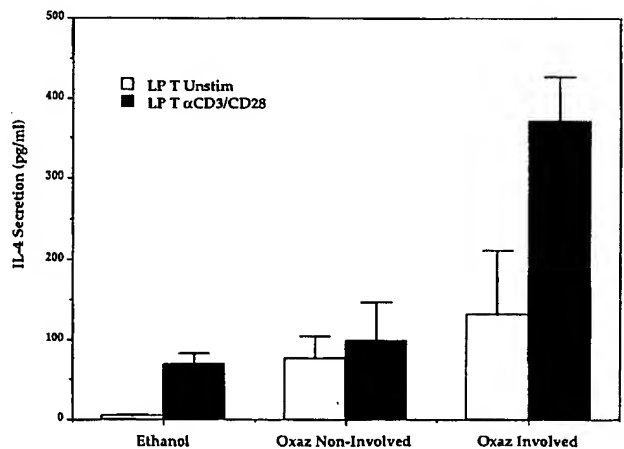


Figure 5. In vitro IL-4 secretion by LP T cells extracted from proximal (noninvolved) and distal (involved) colons of mice with oxazolone-induced colitis as compared with control (ethanol-treated) mice. Data shown represent pooled values from three independent experiments. Standard errors are indicated.

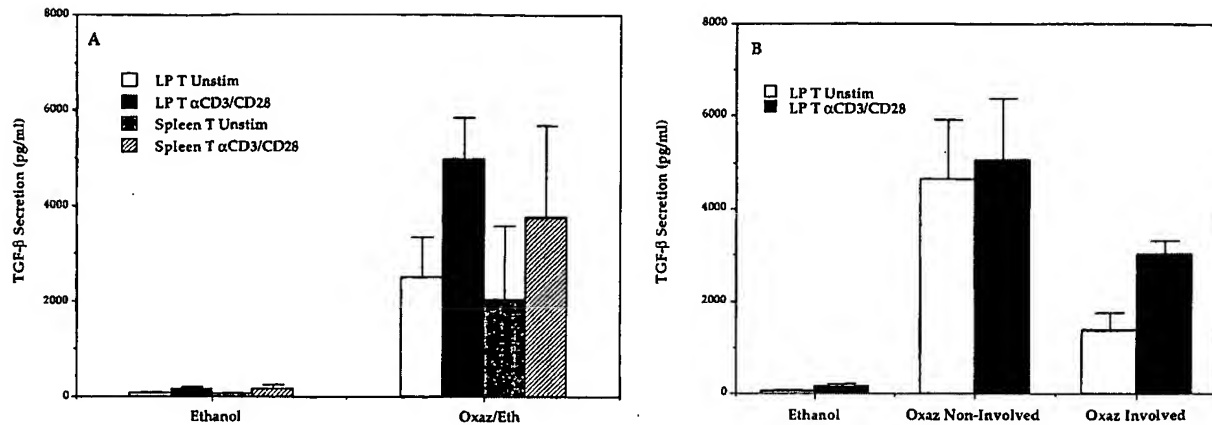


Figure 6. (A) TGF- β secretion of unstimulated and stimulated LP and spleen T cells (LP T cells isolated from whole colons of mice with oxazolone colitis). LP T and spleen T cells were isolated from oxazolone and control (ethanol-treated) mice on day 2, cultured for 48 h in the presence or absence of anti-CD3 and anti-CD28, and culture supernatants were analyzed for concentration of TGF- β secretion by specific ELISA. Data shown represent pooled values from three independent experiments. Standard errors are indicated. (B) In parallel experiments, purified LP T cells were extracted from proximal (noninvolved) and distal (involved) colons of mice with oxazolone colitis and compared with control (ethanol-treated) mice for TGF- β secretion. Data shown represent pooled values from three independent experiments. Standard errors are indicated.

0.01). In addition, as shown in Fig. 6 B, purified LP T cells isolated from the uninflamed proximal colonic tissue produced fourfold more TGF- β in the unstimulated state and almost twofold more TGF- β in the stimulated state than T cells isolated from inflamed distal colon tissue ($P < 0.05$).

Cytokine Production of LP T Cells of Mice with Oxazolone Colitis During the Resolution Phase of the Colitis. In a further series of studies of cytokine production of T cells from mice with oxazolone colitis, we determined the cytokines produced by LP T cells obtained from mice who had survived oxazolone colitis and were in a resolution phase of the inflammation (at 10 d after oxazolone administration per rectum). As shown in Fig. 7, LP T cells from such mice, when cultured in vitro with anti-CD3/CD28, exhibited IL-4 production comparable with that of T cells

from control mice ($P > 0.05$), but exhibited IL-5 production as high as that seen at the peak of the inflammation (i.e., at 48 h) ($P < 0.01$). In addition, while TGF- β production by T cells obtained from the distal colons of mice with resolving colitis compared with that by T cells from colons with acute colitis was diminished, it was still twofold higher than TGF- β production by control LP T cells when cultured in vitro with anti-CD3/CD28 ($P < 0.05$). Finally, T cells isolated from the proximal, noninvolved colons of mice with resolving colitis continued to secrete high levels of TGF- β (282 pg/ml in the unstimulated state, 5,316 pg/ml in the stimulated state).

Treatment of Oxazolone Colitis with Various Anticytokine Antibodies. In a final series of studies we sought to evaluate the role of elevated IL-4 and TGF- β secretion in the

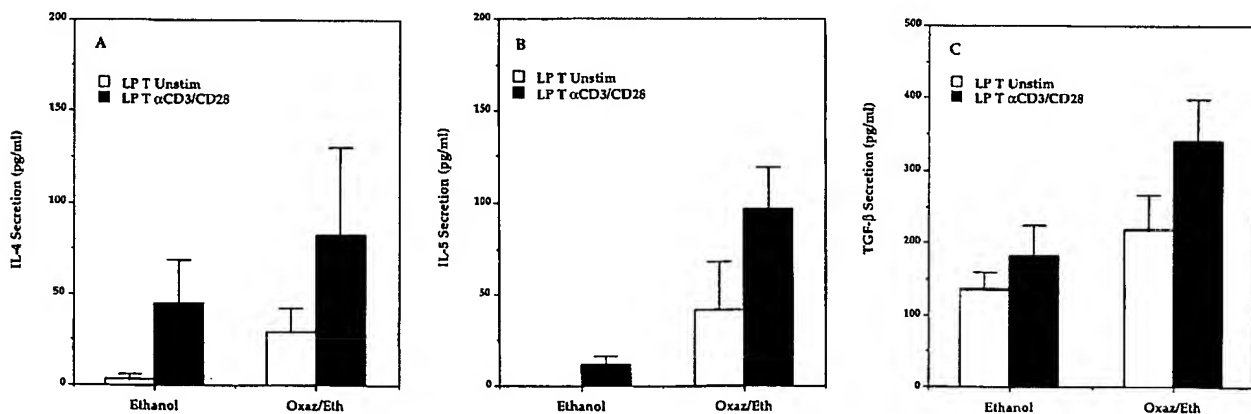


Figure 7. Cytokine production by LP T cells from resolving oxazolone colitis. LP T cells isolated from the distal colons of mice with oxazolone colitis and control ethanol-treated mice at 10 d after intrarectal administration were stimulated in vitro as indicated in the legend to Fig. 4. Culture supernatants were assayed for IL-4, IL-5, and TGF- β by specific ELISA. Data shown represent pooled values from three independent experiments. Standard errors are indicated.

induction and resolution of oxazolone colitis by the systemic coadministration of antibodies to these cytokines and to an inducer of Th1 responses, IL-12. Accordingly, we administered anti-IL-4 antibody (3 mg), anti-TGF- β (1 mg), anti-IL-12 (2 mg), or control rat IgG (1 mg) intraperitoneally to mice at the time of induction of oxazolone colitis with intrarectal oxazolone administration and then noted the effects of these treatments on the course of oxazolone colitis and on cytokine production. As shown in Fig. 8, while the mice that had received anti-IL-4 lost weight during the first 24 h after oxazolone administration, they quickly regained the lost weight and were near baseline weight within 4 d of oxazolone/antibody administration; in contrast, mice that had received anti-TGF- β or anti-IL-12 manifested a weight loss pattern similar to or more severe than that exhibited by the mice that had been administered control IgG.

Macroscopic appearance of the colons of the mice in the various treatment groups is depicted in Fig. 9. While the colons from the anti-IL-4-treated mice were similar in appearance to that of the control ethanol-treated mice, the colons from the anti-TGF- β -treated mice revealed a severe colitis now involving the entire length of the colon, not just the distal half, as in untreated oxazolone colitis. The effects of anti-IL-12 varied; 50% of the mice manifested no change in the distal colitis seen after oxazolone administration whereas the other half displayed a colitis now involving the entire colon.

The above findings concerning the effects of antibody treatment on the development of oxazolone colitis correlated well with antibody treatment-induced changes in cy-

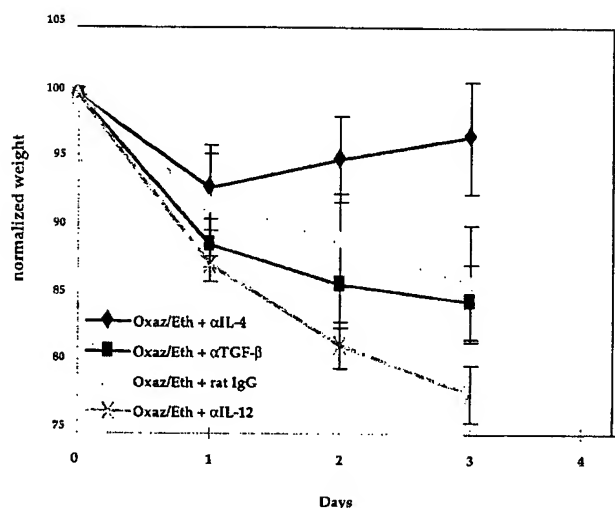


Figure 8. Anti-IL-4 antibodies can prevent the onset of oxazolone colitis. Weight changes in SJL/J mice who received intrarectal oxazolone administration and then treated with either rat IgG control Ab, anti-TGF- β mAb, or anti-IL-12 mAb. After initial reduction in body weight in all groups at day 1, the mice treated with anti-IL-4 showed a significant increase in body weight, whereas mice treated with either IgG control Ab, anti-TGF- β , or anti-IL-12 continued to lose body weight. Each point represents pooled data from five mice. Standard errors are indicated.

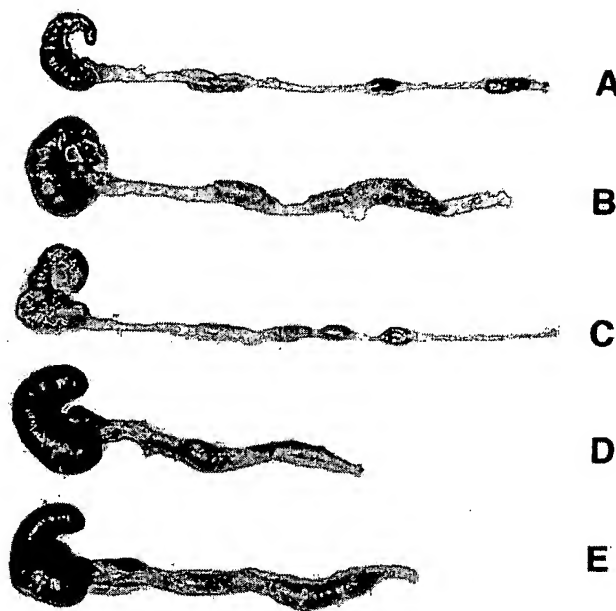


Figure 9. Macroscopic appearance of colons obtained from (A) control (ethanol-treated) mice; SJL/J mice who received intrarectal oxazolone or intrarectal oxazolone plus (B) IgG control Ab; (C) anti-IL-4; (D) anti-TGF- β mAb; or (E) anti-IL-12 mAb (all antibodies were administered intraperitoneally). Mice treated with anti-IL-4 mAb showed a significant resolution in inflammation, whereas mice treated with anti-TGF- β reveal a severe colitis that now involves the entire colon. Colons from one representative experiment out of two are shown.

tokine secretion. Thus, as shown in Fig. 10, administration of anti-IL-4 to mice undergoing induction of oxazolone colitis was, as expected, associated with a great reduction in IL-4 and TGF- β production by isolated colonic (lesional) T cells, as compared with cells obtained from untreated mice or mice administered control IgG. On the other hand, administration of anti-TGF- β to mice undergoing induction of oxazolone colitis was associated with an expected reduction in TGF- β production, but a continued increased level of IL-4 production. However, such treatment had no effect on IFN- γ production, i.e., such production remained undetectable (data not shown). Finally, administration of anti-IL-12 to mice undergoing induction of oxazolone colitis was also associated with enhanced production of IL-4, but no change in TGF- β secretion.

The above "clinical" and cytokine data, taken together, strongly suggest that IL-4 has a primary proinflammatory role in the development of oxazolone colitis, whereas TGF- β has an important counterregulatory role in disease induction and the extent of colon involved with disease.

Discussion

Oxazolone colitis, the murine colitis described here, is a new form of experimental colitis that is easily distinguishable from the colitis produced by the intrarectal administration of another contactant, TNBS, or indeed most other

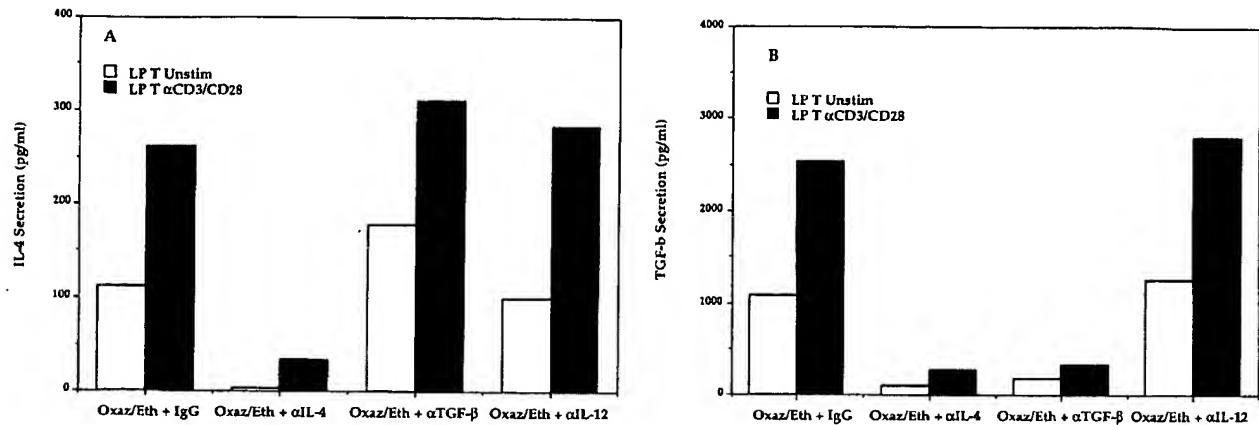


Figure 10. Cytokine production of stimulated and unstimulated LP T cells in oxazolone-induced colitis mice treated with rat IgG control Ab, anti-IL-4, anti-TGF- β mAb, or anti-IL-12 mAb at the time of intrarectal oxazolone administration. LP T were isolated on day 3, cultured for 48 h in the presence or absence of anti-CD3 and anti-CD28, and culture supernatants were analyzed for concentration of (A) IL-4 and (B) TGF- β by specific ELISA. Data from one representative experiment out of two are shown.

forms of experimental colitis. First, it is a rapid onset inflammation that peaks within a few days of oxazolone administration and leads to wasting and bloody diarrhea resulting either in the death of the mouse or complete recovery. Thus, it is different from TNBS colitis, which is a more slowly developing intestinal inflammation that peaks 1 wk after TNBS administration and that tends to persist in surviving mice. Second, the macroscopic and microscopic changes observed in oxazolone colitis and TNBS colitis are very different; oxazolone colitis is marked by inflammation strictly limited to the distal half of the colon, whereas TNBS colitis is a pan-colitic. In addition, at the microscopic level the inflammation of oxazolone colitis is relatively superficial and is characterized by ulceration, a cellular exudate, and a mixed inflammatory infiltrate of lymphocytes, granulocytes, and eosinophils. TNBS colitis, on the other hand, is a full-thickness inflammation which is associated with a dense, lymphocyte/macrophage infiltration occasionally arrayed as a granuloma. Third, and perhaps most importantly, the nature of the immune response in the two colitides are different. In oxazolone colitis the T cell response is an IL-4-driven Th2 T cell response which is marked by elevated IL-4/IL-5 production and normal (low) IFN- γ production and which is prevented by the systemic coadministration of anti-IL-4. In contrast, in TNBS colitis an IL-12-driven Th1 T cell response prevails, marked by an elevated IFN- γ response and an inflammation that is treated by the administration of anti-IL-12 (1). These characteristics of oxazolone colitis and TNBS colitis can be compared with those of human UC and Crohn's disease, the two major forms of human inflammatory bowel disease. Thus, in previous studies conducted by ourselves and others, it has been shown that human UC is associated with elevated IL-5 secretion and normal IFN- γ secretion; nevertheless, it cannot as yet be called a Th2 inflammation because it is not associated with elevated IL-4 secretion, the usual driving force of Th2 responses. On the other hand, established Crohn's disease is quite definitely a Th1 inflamma-

tion since IL-12 and IFN- γ secretion is elevated and there is no increase in either IL-4 or IL-5 secretion (2-5, 31). On this basis, it seems reasonable to suggest that oxazolone colitis is a murine model related to but not identical to UC, whereas TNBS colitis is a model related to Crohn's disease.

Most experimental murine models of colitis studied thus far have been shown to be more like TNBS colitis than oxazolone colitis, i.e., IL-12-driven Th1 T cell-dependent inflammatory responses that, as mentioned, histopathologically resemble human Crohn's disease. This is true of colitis models with quite disparate immune defects such as the colitis occurring in SCID mice reconstituted with normal (naive) T cells, as well as IL-10 and IL-2 knockout mice (32-34). In addition, it is also true of a recently described model of spontaneously occurring intestinal inflammation occurring in the SAMPl/YIT strain of mice that is unique because it involves the small intestine rather than the colon (35). The existence of these various models implies that a number of immunologic conditions occurring in both normal mice and mice with genetic defects of immune function can give rise to a final common pathway: the unregulated production of IL-12 and the resulting development of a Crohn's disease-like intestinal inflammation. Conversely, it implies that the skewed Th1 response occurring in the intestine of patients with Crohn's disease could result from any of several immunologic abnormalities whose only commonality is that they ultimately result in a dysregulated Th1 response.

An important and relevant exception to the above rule that experimental colitides in mice are usually the result of Th1 inflammatory responses is inherent in the characteristics of oxazolone colitis as well as the colitis developing in TCR- α chain knockout mice. Thus, as documented here and as reported previously, in both of these situations an IL-4-driven Th2 colitis develops that histopathologically more closely resembles UC than Crohn's disease (36-38). The existence of these models implies that a number of different immunologic conditions can also lead to another final common pathway, namely the unregulated induction of

Th2 T cells. In addition, these two latter models, when considered in conjunction with the Th1 models, provide additional support for the view that immunologic conditions leading to Th2 and Th1 dysregulation are the basis of UC and Crohn's disease, respectively.

The colitis occurring in TCR- α chain knockout mice, in that it is immunologically similar to oxazolone colitis, bears further discussion. TCR- α chain knockout mice develop T cells with low expression of TCRs comprised of β -chain homodimers. Therefore, their tendency to develop Th2 responses may be related to necessarily aberrant interactions with APCs. The colitis that occurs in such mice is a slowly developing disease that appears to originate in the appendiceal tissue and thus initially involves the cecal area of the colon exclusively; ultimately, however, it involves the entire colon and then persists as a chronic inflammation (36–40). This pattern of inflammation differs from that seen in oxazolone colitis, since the latter is a distal rather than a proximal colitis and is an acute but ultimately self-limited disease (provided the mice survive the period of acute inflammation). The reason for these differences awaits further analysis of the cytokine milieu present in the two models, particularly after the inflammation becomes well established. One possibility, based on the fact that the locus and course of oxazolone colitis probably depend on the nature of the elicited TGF- β response, is that oxazolone colitis and the colitis of TCR- α chain knockout mice are associated with qualitatively and quantitatively different TGF- β responses.

One of the striking features of oxazolone colitis is that it is associated with high LP T cell production of TGF- β that is greater in proximal, uninvolved colons than in the distal, involved colon. This, plus the fact that anti-TGF- β administration to mice at the time of intrarectal oxazolone administration leads to pan-colitis (i.e., involvement of the normally uninvolved proximal colon), strongly suggests that the TGF- β response in the proximal colon prevents disease in this segment of bowel, whereas the response in the distal colon, while still relatively high, is not sufficient to prevent disease in this segment of the bowel; it is thus the colonic TGF- β gradient that explains the proximal distribution of disease in oxazolone colitis. In addition, it seems likely that the short-lived nature of the distal inflammation in oxazolone colitis is attributable to the still relatively high TGF- β response in this area of the colon and thus the ability of the latter response to eventually overcome the distal inflammation. On the basis of these findings in oxazolone colitis, it is reasonable to suggest that the generally distal distribution of inflammation in UC is also due to a TGF- β gradient in the human colons. However, this suggestion runs counter to recent findings showing that TGF- β synthesis is higher in the inflamed areas of the UC colon than in uninfamed areas (41). One possible resolution of this discrepancy lies in the fact that in the study cited TGF- β synthesis was measured at the mRNA level in whole colonic biopsies; thus TGF- β produced by all cells

was measured and the gradient referred to above may only apply to T cell production of this cytokine.

The high TGF- β response in oxazolone colitis contrasts with the virtually nil TGF- β response in TNBS colitis (unless TNBS is concomitantly given by mouth in the form of haptenated protein) (6, 7). These very different TGF- β responses in oxazolone and TNBS colitis are at least partially explained by recent studies of the differentiation of naive T cells into TGF- β -producing T cells in primary and secondary cultures (42). In these studies it was shown that Th1 responses (i.e., IL-12 and IFN- γ production) inhibit the differentiation of TGF- β -producing cells whereas Th2 responses (IL-4 production) favor such differentiation. Whether or not these effects are completely independent is still somewhat unclear, but, in any case, they may explain the fact that a Th2 T cell-induced inflammation such as oxazolone colitis is associated with a high TGF- β response.

One question that remains to be answered concerning the high TGF- β response in oxazolone colitis is why this response did not prevent the inflammation from developing in the first place, in that much lower TGF- β responses induced by the feeding of TNP haptenated-protein to mice prevents the induction of TNBS colitis (6). The answer to this question may lie in the relative ability of TGF- β -producing T cells to suppress Th1 responses and Th2 responses: it is possible that Th2 responses are resistant to TGF- β -mediated suppression, whereas Th1 responses are susceptible to such suppression. Indirect evidence in favor of this possibility is that certain immune responses normally supported by Th2 cytokines such as humoral IgA responses actually require TGF- β and are not suppressed by the latter except at high TGF- β concentrations (43).

The induction of a Th2-mediated colitis by the rectal administration of oxazolone (at the doses used) and a Th1-mediated colitis by the rectal administration of TNBS raises the fundamental question as to the relation of the nature of the stimulating antigen to the course of T cell differentiation. At the moment this question can only be answered in general terms by the suggestion that the initial interaction between mucosal APCs presenting TNBS or oxazolone to T cells recognizing these haptens results in preferential excretion of IL-12 in the case of TNBS and IL-4 in the case of oxazolone. Whether this relates to antigen affinity for available T cell receptors and subsequent patterns of expression of CD40L and/or B7 is unknown and awaits further study. Nevertheless, this dichotomy does suggest that whether or not an individual susceptible to inflammatory bowel disease develops UC or Crohn's disease may to some extent depend on factors relating to the nature of the initial inducing antigen.

In summary, oxazolone colitis is a new mucosal model of colitis that is an IL-4-driven, Th2 inflammation that has features resembling the human disease, UC. This colitis is regulated in a unique manner by TGF- β production, an observation that may have significance to the factors that regulate its human counterpart.

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